Interaction among kidney disease-specific risk factors for cardiovascular disease

van Breda, G.F.

2019

document version
Publisher's PDF, also known as Version of record

INTERACTION AMONG KIDNEY DISEASE-SPECIFIC RISK FACTORS FOR CARDIOVASCULAR DISEASE

.....

Het gaat niet om de bestemming,

maar om de reis ernaartoe

……..

Boeddha

Interaction among kidney disease-specific risk factors for cardiovascular disease

ISBN:	978-94-6375-478-1
Author:	Grietje Fenna van Breda
Layout and design:	Jules Verkade, persoonlijkproefschrift.nl
Printing:	Ridderprint BV I www.ridderprint.nl

The research in this thesis was financially supported by Amgen.
The printing of this thesis was supported by ViforPharma and B-Braun.

VRIJE UNIVERSITEIT

INTERACTION AMONG KIDNEY DISEASE-SPECIFIC RISK FACTORS FOR CARDIOVASCULAR DISEASE

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor
aan de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. V.Subramaniam,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de Faculteit der Geneeskunde
op vrijdag 4 oktober 2019 om 13.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

Grietje Fenna van Breda

geboren te Sassenheim

promotoren:	prof.dr. C.A.J.M. Gaillard
	prof.dr. M.G. Vervloet
copromotor:	dr. M.H. de Borst

Table of Contents

Chapter 1

GENERAL INTRODUCTION AND OUTLINE OF THIS THESIS

Cardiovascular risk factors in chronic kidney disease

Chronic kidney disease is an increasing problem for public health systems. Patients with chronic kidney disease (CKD) have a strikingly elevated risk for cardiovascular complications. The excess cardiovascular risk is proportional to the decrease in renal function and level of proteinuria and most patients with CKD die due to cardiovascular disease (CVD) rather than the kidney disease itself (1). Improving outcomes in patients with CKD therefore does not only require protecting kidney function, but also protecting the cardiovascular system from the consequences of CKD.

Since traditional cardiovascular risk factors, like hypertension, hyperlipidemia and diabetes do not entirely explain this high risk in CKD, there is legitimate interest in exploring non-traditional CKD related risk factors, like proteinuria, vitamin D deficiency, iron deficiency and anemia.

Disturbances of the CKD- mineral bone disease axis (CKD-MBD) occur early in the course of CKD (eGFR < 60 ml/min). Traditionally the early pathogenesis of CKD-MBD has been ascribed to a decline in 1.25 dihydroxyvitamin D (1.25 (OH)2D) levels. Low 1.25 (OH)D levels lead to increase in serum parathyroid hormone (PTH) and alterations in calcium and phosphorus metabolism. The combination of secondary hyperparathyroidism, hyperphosphatemia and vitamin D deficiency was regarded for years as a main causative factor for cardiovascular diseases in CKD (2). However, since the discovery of fibroblast growth factor 23 (FGF23) in 2000 by the ADHR consortium (3) and Klotho by Kuro-O et al. (4) in 1997, this view has dramatically changed as it became clear that high FGF23 concentrations and klotho deficiency may be a putative missing link between CKD-MBD and CVD.

Nowadays, traditional risk factors and disturbances of these CKD related risk factors are key targets for intervention in CKD to prevent CVD. Despite this treatment strategy, the protection against CVD by current therapy is far from satisfying. Current pharmacological treatments to prevent CVD in CKD target pathophysiological processes which are mostly clarified yet. However, unraveled mechanisms and crosstalk between CKD specific risk factors could exist with a possible amplifying risk on CVD (figure 1). Treatment with active vitamin D for instance, instituted to control PTH, may induce FGF23 or hyperphosphatemia. This thesis focuses on the effects of different CKD specific risk factors on each other. It would be attractive to hypothesize that an integrated approach targeting optimization of amplifying risk factors, instead of targeting risk factors in isolation, enhances therapeutic efficiency, either by adapting current pharmacological strategies or by novel interventions.

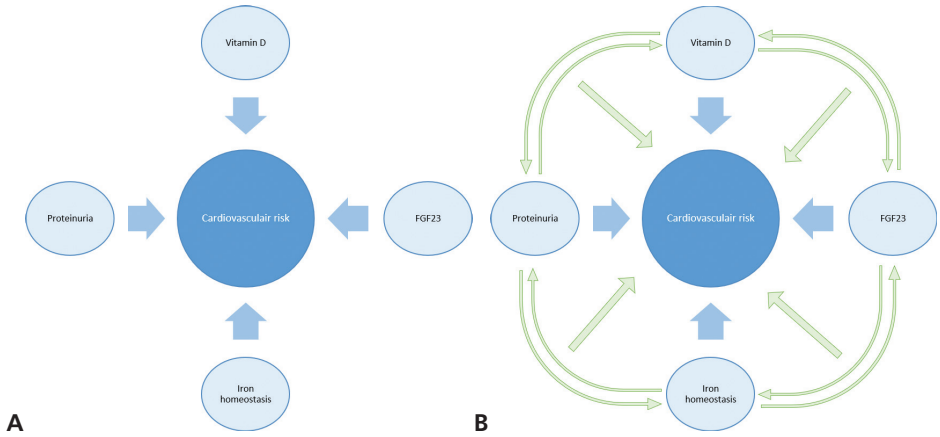


Figure 1. The isolated impact of CKD specific risk factors like FGF23, proteinuria, vitamin D deficiency and iron deficiency on CVD has been investigated last decade (Panel A). Last years, there has become more evidence for amplifying effects among these risk factors (Panel B).

1. Fibroblast growth factor 23 (FGF23) and Klotho as risk factors for CVD in CKD

FGF23 is a hormone produced in osteocytes that controls phosphate and vitamin D homeostasis (5). FGF23 has several primary physiological actions. FGF23 reduces renal phosphate reabsorption by down regulating the luminal expression of sodium-phosphate co-transporters NPT2a and NPT2c in the proximal tubules (6). FGF23 also suppresses circulating levels of 1.25(OH)2D by directly inhibiting the renal enzyme 1-α hydroxylase, responsible for formation of active vitamin D, and stimulating the catabolic 24-hydroxylase in the kidney, which degrades both 25(OH)D and 1.25(OH)2D (7). In addition, FGF23 inhibits the secretion of parathyroid hormone (PTH) (8). Healthy humans and mice are able to maintain their serum phosphate levels in a relatively narrow range regardless of phosphate intake, in part because circulating FGF23 levels adapt to phosphate intake (9-11). Patients with CKD however, have sustained increases of serum FGF23 concentrations compared with healthy individuals due to stimulation by CKD related abnormalities in bone and mineral metabolism like hyperphosphatemia, hypo- and hypercalcemia and hyperparathyroidism. Elevated FGF23 is the earliest detectable serum abnormality of CKD-MBD (12) and its rise occurs before changes in serum concentration of PTH, 1.25(OH)2D or even phosphate are detectable (13). The highest levels of FGF23 are seen in end-stage kidney disease: there is an exponential increase in FGF23 levels as glomerular filtration rate (GFR) declines, reaching up to 800-fold increased concentrations in patients with end stage kidney disease, compared with healthy individuals (13). Beside adaptive FGF23 production due to CKD-MBD abnormalities, decreased renal clearance contributes to the increased FGF23 serum concentrations in patients with CKD.

FGF23 circulates as intact (full length polypeptide) FGF23 (iFGF23) and can be cleaved into n-terminal and c-terminal fragments (cFGF23) (14). The phosphaturic activity is exerted only by iFGF23, thus its biologic effect reflects the equilibrium between production and degradation of the mature hormone. In patients with end stage kidney disease (ESKD), iFGF23 appears to be the primary circulating form (15). To get insight into the degree of proteolytic degradation of FGF23 and factors that influence FGF23 production, cleavage and clearance, it is important to measure iFGF23 and cFGF23 simultaneously in peripheral blood samples. Circulating FGF23 concentrations can be quantified by commercially available ELISA (fig 2b). Intact FGF23 assays (iFGF23) measure exclusively biologically active FGF23 because the capture antibodies recognize two epitopes that flank the proteolytic cleavage site, as shown in figure 2b (16). C-terminal FGF23 (cFGF23) detects both intact FGF23 and its C-terminal fragments because the capturing antibodies recognize two epitopes that both reside at the C-terminus (17).

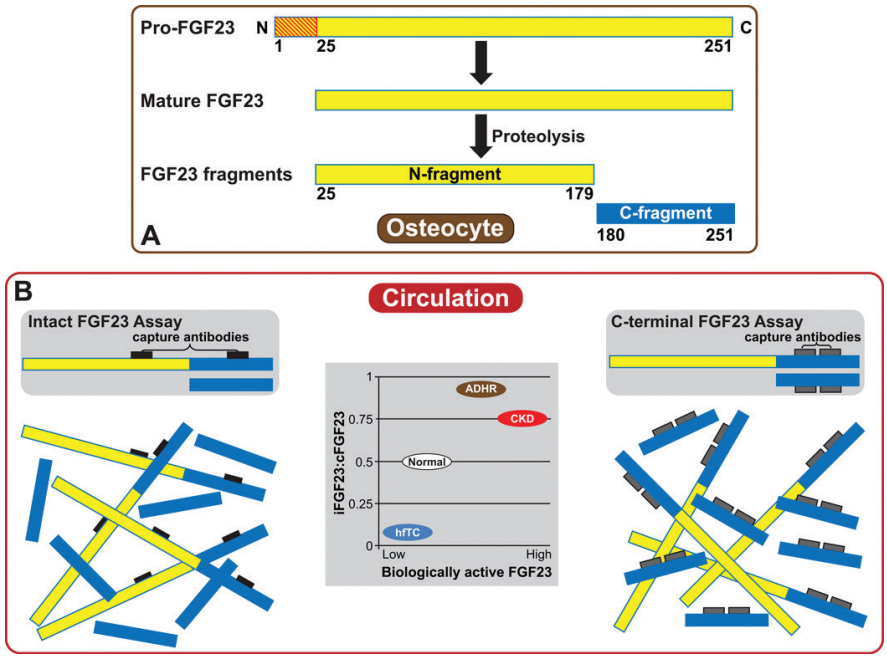


Figure 2. Schematic of FGF23 processing and strategies to quantify circulating FGF23 levels, adapted from Wolf et al.(18) A: FGF23 is expressed in osteocytes as a 251-amino acid protein. A 24-amino acid N-terminal signaling peptide (red striped) is cleaved to form the mature molecule, which can then be secreted as either intact or cleaved protein. This cleavage then occurs within osteocytes between amino acids 179 and 180 to form inactive N-terminal and C-terminal fragments. **B**: Two types of commercially available ELISAs detect FGF23 using different molecular strategies. Intact assays use capture antibodies (black) that bind two epitopes that flank the proteolytic cleavage of FGF23 site at both sides, so that only biologically active, intact FGF23 (amino acids 25-251) is detected. C-terminal assays use capture antibodies (gray) that bind two epitopes on the C-terminus of FGF23. This assay does not distinguish biologically active, intact FGF23 from inactive C-terminal fragments (amino acids 180-251).

A second important protein involved in CKD and CVD is Klotho. Klotho was originally identified as an anti-aging gene: mutation of the gene encoding for Klotho, that induced a downregulation of its transcript, led to a premature aging-like syndrome in mice (4). Klotho is predominantly produced in the distal tubular epithelial cells of the kidney, but also in the proximal tubule albeit at much lower amounts (19). Klotho is essential for FGF23 signal transduction by increasing the affinity of the FGF receptor type 1 for its ligand FGF23. Already in the early stages of CKD, klotho levels decrease and this progresses with further renal function loss (20). Declined Klotho levels are partly caused by loss of renal tissue but the uremic environment with increased inflammation and oxidative stress has negative impact as well (21). Lower Klotho levels in turn are associated with more rapidly progressive CKD (22), CVD (23), vascular calcification (24) and increased mortality (25). Hu et al. showed that downregulation of Klotho in CKD plays a pathogenic role in the progression of CKD and induces vascular calcification: transgenic mice that overexpressed Klotho had lower levels of proteinuria, better renal function and less vascular calcification compared with wild-type mice with CKD. Conversely, Klotho knockout mice with CKD had worse renal function and severe calcification (26).

1.1. Direct effects of FGF23 on cardiovascular risk in CKD

During the past decade, numerous observational studies have described the association between FGF23 and main adverse clinical outcomes in CKD: progression to ESKD, CVD and death (27-29). Even in the general population, FGF23 levels have been associated with increased cardiovascular risk (30). Despite these epidemiological studies showing the close relationship between FGF23 and CVD, there is only limited experimental studies pointing to a causal relationship. A main experimental study performed by Faul et al. (31) demonstrated that FGF23 may be directly involved in the development of left ventricular hypertrophy. Additional evidence for the role of FGF23 in LVH came from an observational study performed by Baia et al. In patients who underwent a kidney transplantation, FGF23 was significant associated with cardiovascular mortality even after adjustment for several major cardiovascular risk markers, demonstrating that FGF23 is a strong and independent risk factor (32). Remarkable was the independent association of FGF23 with markers of heart failure (MR-proANP, NT-proBNP and copeptin). However, modulation of the association between FGF23 and cardiovascular mortality by these cardiac markers could not be demonstrated. A recent meta-analysis of 19 cohort studies postulated that FGF23 could initiate and exacerbate cardiovascular diseases and is a possible contributor to CKD progression and CVD development (33).

Based on the aforementioned literature, it is tempting to target therapies to lower FGF23 to improve cardiovascular outcomes. However, it is important to realize the beneficial effects of

FGF23, like maintaining phosphate levels in narrow ranges. Theoretically, there can be a distinct pathological effect of cFGF23 and iFGF23. As there is much literature on the physiological function of iFGF23 , data regarding actions of FGF23 fragments are scarce (34). Rygasiewicz et al. (35) identified cFGF23, but not iFGF23, as a predictor of incident acute kidney failure (AKI) and death in critically ill patients, even after correction for baseline renal function and incident AKI. They postulate cFGF23 as a novel biomarker for AKI and death. In a mouse model of phosphate wasting disorders, Goetz et al. demonstrated that cFGF23 was effective in counteracting the phosphaturic action of FGF23 by a dual mechanism: it downregulates FGF23 binding site for the binary FGFR-Klotho complex, and in addition acts as an endogenous competitive inhibitor of FGF23 (34). That cFGF23 instead of iFGF23 may be the culprit in CVD risk was confirmed by study results in renal transplant recipients (RTR) (36). Both iFGF23 and cFGF23 plasma levels and iron deficiency (ID) status were determined in 700 stable RTRs. In multivariable-adjusted Cox regression analyses, ID was associated with increased mortality. Further analysis showed that 46% of the association between ID and mortality was explained by cFGF23 and none by iFGF23. They concluded that cFGF23 fragments may be an important mediator of the association between ID and mortality. However, the question remains whether this association between cFGF23 fragments and dismal outcome is a reflection of mechanisms that drive the intensity of intact molecule cleavage or that the FGF23 fragments have biological effects by itself.

Based on all these studies, it seems obvious that high FGF23 levels are a risk factor for cardiovascular disease and death in patients with CKD. However, despite increased understanding of the biology of FGF23, fundamental aspects of the regulation in health and in CKD and the role as causative factor for cardiovascular disease still remains unknown.

1.2. Indirect effects of FGF23 on cardiovascular risk in CKD

Recently, it was shown that FGF23 exerts effects beyond mineral metabolism, being involved in erythropoiesis, infection rates and iron metabolism (37). Experimental animal studies have been published expanding our understanding regarding interaction of FGF23 and erythropoiesis. Genetic deletion of FGF23 in mice resulted in increased erythropoiesis and administration of exogenous FGF23 in wild-type mice decreased erythropoiesis, independent on vitamin D levels (38). Hypoxia inducible factor-proline hydroxylase (HIF-PH) inhibitors act as a hypoxia mimetics that stabilize HIF, thereby inducing the transcription of endogenous erythropoietin and erythropoiesis (39). Additionally, HIF-PH inhibitors modulate the uptake and availability of iron (40). David et al. demonstrated the increase of FGF23 plasma levels after treatment with hypoxia inducible factor-proline hydroxylase (HIF-PH) inhibitors (39). Mehta et al. performed a prospective cohort study in which the role of elevated FGF23 levels as a risk factor for development of anemia was determined

(41). They showed a significant association of high FGF23 levels with prevalent anemia, decline in hemoglobin levels over 4 years and risk of incident anemia after adjustment for other CKD risk factors and mineral metabolism markers. Recently, the association between high FGF23 levels and development of anemia in a CKD mouse model was investigated by blocking the effects of FGF23. The authors revealed that inhibiting FGF23 signaling stimulated erythropoiesis and abolished anemia and iron deficiency (42). In summary, these studies suggest that elevated FGF23 levels are a causative factor in the development of renal anemia and iron deficiency. Beside the interactions of FGF23 with erythropoiesis and HIF-PH, there may several other explanations for the association between FGF23 and anemia. First, high FGF23 levels may be a marker of declined kidney function and chronic inflammation: both can cause anemia, in which case the association between FGF23 and anemia is just a confounder. Second, the negative effect of high FGF23 levels on hemoglobin levels may be mediated by FGF23 induced reduction of active vitamin D because low vitamin D levels are associated with anemia (43). Third, there is growing evidence for the role of iron on FGF23 regulation (see paragraph 2). Although the link between iron, FGF23 and erythropoiesis appears relevant, complete characterization of these complex interactions needs further studies.

Additionally, several studies suggest that FGF23 increase RAAS activation and therefore, ACEi therapy is less effective in CKD patients with high FGF23 levels (44). Humalda et al showed that high FGF23 levels were associated with an impaired response to sodium restriction in addition to ACE-inhibition in patients with CKD (45). Furthermore, in patients with new-onset and worsening heart failure, higher FGF23 levels were associated with less successful uptitration of ACEi/ARB's and an increased risk of all-cause mortality and HF hospitalization (46).

2. *Iron deficiency is a risk factor for CVD in CKD*

Iron deficiency is a common and clinically relevant problem in patients with CKD. Iron deficiency may have several explanations: insufficient dietary iron uptake is provoked by poor appetite and dietary restrictions and intestinal bleeding may result in increased iron losses (47). Additionally, patients undergoing hemodialysis (HD) experience significant additional iron losses due to blood remaining in the dialyser circuit after treatment. In the context of anemia, the role of hepcidin has been subject of research for the last decade. In this patient population, chronic inflammation leads to increased hepcidin production in the liver. The peptide hormone hepcidin interacts with the cellular iron exporter ferroportin and is the key regulator of iron homeostasis. Ferroportin is the major cellular iron exporter in the cell membrane of hepatocytes, enterocytes and macrophages and is internalized and degraded by hepcidin (48), resulting in increased intracellular iron stores, decreased dietary iron absorption and decreased iron concentration in the circulation. Hepcidin

synthesis is regulated by several physiologic processes. Conditions in which iron demand is increased, like iron deficiency, hypoxia and administration of erythropoietin (EPO), induce a decrease in hepatocellular hepcidin synthesis, thereby promoting iron availability. Iron overload, inflammation and infection on the other hand induce an increase in hepcidin synthesis that leads to impaired iron availability for erythropoiesis. Generally, serum hepcidin levels in CKD patients are elevated (49, 50), partly because the kidney is the main organ responsible for hepcidin clearance (51) and partly because CKD is a state of micro-inflammation, which promotes its hepatic production due to increased cytokine levels. Clinically, this results in diminished iron availability over time leading to the so-called 'anemia of chronic disease'. CKD patients receiving erythropoiesis-stimulating agents (ESAs) to increase hemoglobin levels are very prone to iron deficiency and iron deficiency is the most commonly identified factor of ESA hyporesponsiveness (KDIGO guideline on anemia, 2012). As a result, iron therapy is the cornerstone of treatment, with or without ESAs, in patients with renal anemia.

To gather information about the etiology of 'anemia of chronic disease' in clinical medicine, complete blood count is routinely performed, including red cell distribution width (RDW). Although the normal volume of red blood cells varies from 80 to 100 fL in the blood, a number of physiological conditions may impair erythropoiesis and hence promote a higher degree of heterogeneity of RBC volumes resulting in appearance of smaller (i.e. <60 fL) and larger (up to 120 fL) elements, known as anisocytosis (52). RDW is defined as the standard deviation of erythrocyte volume divided by the mean corpuscular volume (MCV) (i.e., RDW= SD/MCV) and results are routinely expressed as percentages with physiological ranges of values varying between 11 and 15%. Historically, RDW has been used as a marker of iron deficient anemia. Beside used as an aid in the diagnostic work-up for anemia, multiple studies showed that RDW is a robust marker of adverse clinical outcomes in patient with chronic and acute heart failure (53-56), coronary artery disease (57), acute kidney injury (58), HD (59) and even in the community (60, 61). The mechanism underlying the association between RDW and adverse outcome in CKD patients is still unknown, but could be related to the fact that renal dysfunction may be linked with many other risk factors that can elevate RDW levels, like nutritional deficiency, disturbed iron metabolism, oxidative stress or inflammation (56, 62). These factors may be in the causal path to adverse outcome.

2.1. Direct effects of iron deficiency on cardiovascular risk in CKD

The benefits of iron treatment are well-known in patients with chronic heart failure (CHF) and iron deficiency (63-65). In the FAIR-HF trial, treatment with intravenous ferric carboxymaltose (FCM) in patients with chronic heart failure and iron deficiency improved symptoms, functional capacity and quality of life (63). Where cardiologists recognize iron deficiency as a distinct entity regardless

of anemia, nephrologists still treat iron deficiency only in the context of anemia management. Consequently, in contrast to CHF patients, the evidence for detrimental effects of iron deficiency on comorbid conditions associated with CKD is almost non-existent except for anemia, of which iron deficiency is an established contributing factor. In CKD patients, it is well known that anemia is associated with poor quality of life, increased cardiovascular risk and mortality (66-71). Few observational studies investigated the long-term outcome of iron administration beyond its effect on anemia and these studies reported inconsistent results in HD patients (72, 73). To evaluate the impact of iron dextran administration on the survival and rate of hospitalization, 5833 HD patients were included in a multivariable analysis (72). The authors concluded that prescribing > 1000 mg iron dextran over a period of 6 months was associated with a significant elevated rate of death and hospitalization. In addition, prescribing < 1000 mg iron dextran wasn't associated with better outcome. However, they subsequently conducted a cohort study among 32566 HD patients and applied multivariable models to correct for confounding (73). They concluded that the relationship between iron dosing and mortality in HD patients was confounded by incomplete representation of iron dosing and morbidity over time. Kuo et al. performed a prospective cohort study to assess the effectiveness and safety of iron administration in patients with severe kidney failure who not yet started dialysis (74). 31971 patients were divided in two groups with or without iron supplementation within 90 days after starting ESA therapy. After propensity score matching the patients who used iron had a lower risk of all-cause death and hospitalizations, but had higher risk of progression to end-stage renal disease. To date, in contrast to CHF patients in which iron deficiency seems a relevant comorbidity and accounts for poor outcome, the survival benefit of iron supplementation in CKD patients remains largely undefined. More translational research and randomized trials are needed to unravel the exact impact of iron deficiency, and its restoration, on clinical outcomes in CKD patients.

2.2 Indirect effects of iron on cardiovascular risk in CKD: link between FGF23 and iron/anemia

The first clue that iron could be important in the biology of FGF23 came from studies concerning autosomal dominant hypophosphatemic rickets (ADHR). This disorder is the prototype of primary FGF23 excess (3). Due to a mutation in FGF23 gene, the cleavage region on the protein is resistant to proteolytic cleavage, resulting in secretion of primary full-length biologically active FGF23 (75, 76). Due to the resultant high iFGF23 levels, ADHR is characterized by renal phosphate wasting, hypophosphatemia, inappropriately low levels of 1.25(OH)2D and vitamin D-resistant rickets (77, 78). Remarkable is that ADHR has an incomplete penetrance and variable age of onset at birth or later in life (78). Clinical flares of ADHR often coincide with periods of iron deficiency, like puberty,

menses, pregnancy and post-partum period (78). This suggests that iron is an additional regulator of FGF23 beyond classic feedback loops.

Two subsequent experimental mice studies uncovered the role of iron in FGF23 regulation (79, 80). Farrow et al. studied the role of iron status in development of the ADHR phenotype by placing wild type (WT) mice and ADHR knock-in mice on control or low iron diets (79). Both the WT and ADHR mice receiving low-iron diet had significantly increased bone FGF23 mRNA expression compared to the mice with a normal iron diet. Interestingly, wild-type mice with low iron diet maintained normal serum iFGF23 and phosphate concentrations, but had elevated cFGF23 levels. ADHR knock-in mice that received low iron diet manifested elevated iFGF23 levels in addition to elevated cFGF23 levels. Consequently, the iron deficient ADHR mice developed hypophosphatemia and osteomalacia. In WT mice, a second level of FGF23 control is suggested, whereby within osteocytes mature FGF23 protein is cleaved to maintain normal circulating levels of biologically active FGF23 with consequently normal phosphate homeostasis during low and normal iron diet.

The confirmation that iron has an important role in FGF23 regulation in human as well, came from a randomized trial in women with iron deficient anemia due to heavy uterine bleeding who were randomized among intravenous elemental iron in the form of FCM or iron dextran (81). At baseline, iron deficient women showed a similar biochemical profile as iron deficient wild type animals: normal levels of serum phosphate, calcium, 1.25(OH)2 vit D, PTH and iFGF23, but markedly increased cFGF23 levels. Following intravenous iron supplementation, cFGF23 levels decreased whereas iFGF23 levels increased or remained stable, depending on different iron formulations. The exposure of ferric carboxymaltose, but not iron dextran, transiently mimicked the biochemical profile of ADHR mice: increase in iFGF23 levels, hypophosphatemia, reduced 1.25(OH)2 vit D and calcium levels, increased urinary phosphate excretion and secondary hyperparathyroidism. Currently it is unknown why the increase in iFGF23 is only observed after iron supplementation with carboxymaltose iron formulations but not with iron dextran. Despite several studies investigating the effects of administration of intravenous saccharated ferric oxide and iron polymaltose on FGF23-mediated phosphate wasting syndromes (82-86), the pathophysiological process is still not understood. In summary, both animal and human studies demonstrate that iron deficiency stimulates FGF23 transcription in osteocytes which is counterbalanced by increased cleavage of biologically active iFGF23 into inactive cFGF23 fragments within healthy osteocytes, which are then released in to the circulation. However, the exact mechanism by which iron therapy influences FGF23 metabolism, and whether this affects phosphate homeostasis, and why different iron preparations cause more or less hypophosphatemia, remains unclear.

3. Targeting vitamin D deficiency as a risk factor for CVD in CKD

Vitamin D is synthesized in the skin or obtained from nutritional sources and is transported to the liver, where it is metabolized to 25-hydroxyvitamin D (25(OH)D) by the enzyme 25-hydroxylase (CYP2R1). 25(OH)D is the main circulating form of vitamin D and is delivered to the kidney. The second hydroxylation takes place mainly in the kidney, where the 1α-hydroxylase enzyme (CYP27B1) converts 25(OH)D to the active hormone 1.25-dihydroxyvitamin D (1.25(OH)D, calcitriol). 1,25(OH)D acts through the vitamin D receptor (VDR), a nuclear receptor which is expressed ubiquitously in human tissues including the intestine, kidney, parathyroid gland, bone, myocardium, immune system and smooth muscle (87). In CKD, vitamin D deficiency is common, mainly attributed to insufficient renal conversion of 25(OH)D by downregulation of 1 α -hydroxylase activity due to elevated FGF23 levels, but also by insufficient sunlight exposure, nutritional deficits and urinary vitamin D loss by proteinuria (88). Additionally, FGF23 induces 24-hydroxylase (CYP241A) expression which is responsible for catabolization of 25(OH)D by converting it to the inactive 24,25(OH)2D.

3.1. Direct effects of vitamin D on cardiovascular risk in CKD

Vitamin D deficiency has been identified as a risk factor for cardiovascular diseases (89). Experimental studies in vitamin D receptor knockout mice and 1α-hydroxylase knockout mice showed that vitamin D serves as an important factor to maintain blood pressure homeostasis and protecting the cardiovascular system by serving as a negative endocrine regulator of the renin-aldosteron-system (90, 91). Several observational studies have demonstrated the relationship between vitamin D deficiency and poor cardiovascular outcomes in patients with CKD (92, 93). However, the potential benefits of VDRA treatment for this population is controversial. In the PRIMO randomized controlled trial CKD patients with mild to moderate left ventricular hypertrophy (LVH) received oral paricalcitol 2 ug/d or placebo to investigate the effects of paricalcitol on cardiac outcomes. After a treatment period of 48 weeks paricalcitol did not alter left ventricular mass index or improve measures of diastolic dysfunction (94). These results were confirmed in the OPERA trial: 52 weeks of treatment with oral paricalcitol 1 ug/d in patients with severe CKD and frank LVH did not alter measures of left ventricular structure and function (95). Lu et al. performed a systematic review and meta-analysis to assess whether VDRA treatment alters the cardiovascular mortality in patients with CKD (96). They included 17 randomized controlled trials (RCTs) and 21 observational studies. In the observational studies, VDRA treatment was significantly associated with reductions in cardiovascular mortality but this was not confirmed in the RCTs. Thus, prospective studies could not demonstrate a reduction in mortality risk by VDRA treatment, whereas these studies did reveal hypercalcemia as an important safety signal.

3.2. Indirect effects of vitamin D on cardiovascular risk in CKD

Recent clinical observations suggest a possible role of vitamin D in erythropoiesis (97). The administration of analogues of vitamin D has been associated with an improvement of anemia and reduction in erythropoietin requirements (98, 99). Several hypotheses have been postulated to explain this observation. Vitamin D has a suppressive effect on the iron regulatory protein hepcidin, allowing iron absorption and release from storage sites thereby promoting erythropoiesis (100). Another explanation could be that PTH directly inhibits erythroid precursors, EPO synthesis and red blood cell survival (101), while active vitamin D suppresses PTH thereby attenuating the detrimental effects of PTH. However, there is still concern about the supplementation of vitamin D to correct anemia in patients with CKD: no RCT's are available that have evaluated the effects of 25(OH)D or 1,25(OH)D on hemoglobin concentrations as main outcome parameter in CKD (102).

Besides potential effects on anemia, additional arguments to initiate vitamin D treatment in CKD comes from accumulating evidence that vitamin D has renoprotective effects by reducing proteinuria. As mentioned above, proteinuria is a major risk factor for adverse renal and cardiovascular outcomes in CKD (103, 104). The cornerstone of proteinuria treatment is sodium restriction and pharmacological blockade of the renin-angiotensin-aldosterone system (RAAS) by angiotensin converting enzyme inhibition (ACEi) or angiotensin receptor blockade (ARB). RAAS plays an important role in the regulation of blood pressure, electrolyte and volume homeostasis. Monotherapy with ACEi or ARB decrease proteinuria with 20-30% (105) so despite treatment, many patients have residual proteinuria. Combined use of ACEi and ARB seems an efficient strategy to further decrease proteinuria, but safety issues like hyperkaliemia prevent implementation of this dual blockade treatment (106). To improve cardiovascular and renal outcome in patients with CKD, novel anti-proteinuric therapies are being searched for. Currently, there is great interest in the role of treatment with active vitamin D on proteinuria by means of its anti-RAAS, anti-fibrotic and anti-inflammatory effects. Several mechanistic and clinical studies suggest that vitamin D has a suppressive effect on renin transcription. The first clinical studies suggesting an inverse relation between vitamin D and renin levels were published more than 2 decades ago (107, 108). More recently, De Zeeuw et al. (109) performed a RCT that showed that 2 ug paricalcitol (but not 1 ug paricalcitol), given in addition to RAAS blockade, further reduced albuminuria compared with RAAS blockade alone in patients with diabetic nephropathy, particularly in those with high dietary sodium intake. However, it was unclear whether this beneficial effect of paricalcitol was obtained by an effect on renal renin activity. A meta-analysis of available RCT's performed by de Borst et al (110) showed that active vitamin D analogs (mostly added to standard RAAS blockade) achieved a statistical significant reduction in residual proteinuria. In conclusion, beside the role of vitamin D in mineral metabolism, it may interact with other kidney hormones like erythropoietin and renin.

The administration of active vitamin D analogues in combination with optimized RAAS-blockade could be a therapeutic option to reduce the risk of CVD in CKD patients.

Finally, the positive effects of vitamin D in patients with CKD could also be related to the suppression of PTH. High PTH levels are an eminent stimulus for FGF23 production and intervention in this vicious cycle by VDRA administration might have beneficial effects. With the knowledge of the direct interaction of vitamin D and FGF23 on each other and the direct suppressive effect of vitamin D on renin the question raises whether there is a feedback loop between vitamin D-FGF23-klotho axis and RAAS axis.

4. *Proteinuria as a risk factor of CVD in CKD*

Proteinuria is a hallmark of CKD and an important predictor of renal disease progression and cardiovascular complications. The underlying mechanism through which proteinuria affects kidney function seems to be related to tubulotoxic effects of these proteins itself (111). Within the kidney, increased glomerular filtration of protein increase the exposure of tubular cells to excessive protein reuptake in proximal tubular cells. This in turn, leads to activation of multiple pathways that cause the release of vasoactive, inflammatory and fibrotic substances. These processes together result in tubulointerstitial damage and declined nephron functionality (112).

4.1. Direct effects of proteinuria on cardiovascular risk in CKD

The strong predictive value of proteinuria for renal function loss and CVD has been firmly established (113-115). As aforementioned (see paragraph 3.2) the cornerstone of treatment of proteinuria is RAAS blockade, besides sodium restriction. Clinical trials have consistently shown the beneficial effects of this pharmacological intervention: it decreased proteinuria and blood pressure with subsequently decrease in kidney function decline and lowered the risk for CVD (116-118). The pathophysiologic link between proteinuria and cardiovascular risk remains unexplained.

4.2. Indirect effects of proteinuria on cardiovascular risk in CKD

In addition to the demonstrated direct effects of proteinuria on cardiovascular risk in CKD patients, the amount of proteinuria may also partly maintain the cascade of amplifying risk factors. Rat models have shown that massive proteinuria is a risk factor for vitamin D deficiency: vitamin D binding protein (DBP) is lost in urine with consequently low vitamin D levels in serum (119, 120). In a post-hoc analysis of the REIN study, increase in serum phosphate levels turned out to be an independent risk factor for disease progression in patients with proteinuric chronic nephropathies and this high phosphate burden attenuated the renoprotective effects of ACE

inhibitory therapy (121). Consequently increased FGF23 levels may be the culprit of this blunted effect: FGF23 stimulates its FGF23 receptor which increases the production of the converting enzyme with activation of the RAS. Direct and FGF23 mediated inhibitory effects of phosphate on nitric oxide (NO) production might also contribute to limited effects of ACEi therapy mediated bij NO activation. Further evidence for a relationship between FGF23 and proteinuria came from an analysis of 604 patients with moderate to severe kidney disease who participated in the Masterplan study (122). After correction for several well-known cardiovascular and renal risk factors there was a significant association between FGF23 and proteinuria. To shed more light on this association, De Seigneux et al investigated the hypothesis whether proteinuria indirectly modulates kidney phosphate handling via a toxicity that decrease Klotho expression with consequently decreased FGF23 signaling, or via increased proximal tubule phosphate reabsorption (123). At first, they studied phosphate handling in nephrotic children: FGF23 concentration, plasma phosphate and tubular reabsorption of phosphate increased during the proteinuric phase compared with the remission phase. They confirmed this finding by a cross sectional analysis of a cohort of 1738 patients with CKD which showed that proteinuria was predictive of higher FGF23, phosphate and PTH levels. To understand the biological mechanism behind this observation, they developed two animal models with glomerular proteinuria: both nephrotic rats and mice displayed higher renal protein expression of the sodium-phosphate co-transporter NaPi-IIa, lower renal Klotho expression, and decreased phosphorylation of FGF receptor.

In conclusion, there is growing evidence that proteinuria induces elevation of both plasma phosphate and FGF23 concentration, potentially contributing to increased risk of cardiovascular diseases in CKD.

Summary

Altogether, despite the amount of research and the resulting treatment options, patients with CKD still have a high risk of cardiovascular diseases. Currently, the treatment of CKD specific risk factors focus on the treatment of these risk factors separately while there is growing evidence for interactions between these risk factors. CKD specific risk factors should therefore not be considered as separate factors, but they should be examined in their entirety.

AIMS OF THE THESIS

The studies in this thesis aim to unravel cross-talk among novel risk factors for CVD in CKD, and aim to delineate how this cross-talk might amplify the CVD risk. The combined studies should shed more light on future interventions to prevent cardiovascular complications in patients suffering from CKD.

OUTLINE OF THE THESIS

The assumption that iron influences FGF23 cleavage is conceivable on the basis of currently available animal and human studies. In the search for the role of iron on cardiovascular risk factors for CVD, we assessed in **Chapter 2** whether there is a relation between RDW and FGF23 cleavage in patients with both chronic kidney disease and heart failure. Both RDW and FGF23 are independently associated with adverse outcome measures in patients with a cardiorenal syndrome and we tested if iron could be the missing link between these two risk factors.

The role of circulating hepcidin in the etiology of anemia in patient with CKD seems largely elucidated. However, there is increasing evidence that local hepcidin expression in organs like the heart is regulated independently. The role of iron in this local regulation of hepcidin expression is currently unknown. In **Chapter 3** we studied the role of iron in cardiac hepcidin expression in rats with heart failure, renal failure or both.

There is growing evidence for the strong relationship between iron and FGF23 physiology. The underlying biological process driving production and cleavage of FGF23 is still discussed. In the mice study described in **Chapter 4**, we investigated the role of different iron conditions on the metabolism of FGF23. Additionally, the influence of iron on the sensitivity for exogenous FGF23 was tested.

As mentioned before, renal anemia is partly caused by increased hepcidin expression and consequently functional iron deficiency. Several observational and experimental data suggest

that vitamin D deficiency might be an additional co-factor of renal anemia. Potential links between the vitamin D system and erythropoiesis are described in **chapter 5**.

Albuminuria is considered to be an important target for intervention in CKD to slow its progression. Despite optimal pharmacological blockade of the RAAS, residual albuminuria often remains present and is a major risk factor for adverse renal and cardiovascular outcomes in CKD. As vitamin D works as a renin inhibitor, supplementation of vitamin D could decrease the amount of proteinuria. In **chapter 6** the study protocol of the VIRTUE study is presented. The rationale behind this study was to investigate the role of vitamin D receptor antagonist (VDRA) paricalcitol in combination with dietary sodium restriction to provide further albuminuria reduction in non-diabetic CKD patients op top of single RAAS-blockade. In **Chapter 7** the results of the VIRTUE study are presented.

References

1. Saran R, Li Y, Robinson B, Ayanian J, Balkrishnan R, Bragg-Gresham J, et al. US Renal Data System 2014 Annual Data Report: Epidemiology of Kidney Disease in the United States. Am J Kidney Dis. 2015;66(1 Suppl 1):Svii, S1-Svii,305.

2. Wolf M, Thadhani R. Vitamin D in patients with renal failure: a summary of observational mortality studies and steps moving forward. J Steroid Biochem Mol Biol. 2007;103(3-5):487-90.

3. Autosomal dominant hypophosphataemic rickets is associated with mutations in FGF23. Nat Genet. 2000;26(3):345-8.

4. M K-o, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, et al. Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature. 1997;390(6655):45-51.

5. Riminucci M, Collins MT, Fedarko NS, Cherman N, Corsi A, White KE, et al. FGF-23 in fibrous dysplasia of bone and its relationship to renal phosphate wasting. J Clin Invest. 2003;112(5):683-92.

6. Shimada T, Urakawa I, Yamazaki Y, Hasegawa H, Hino R, Yoneya T, et al. FGF-23 transgenic mice demonstrate hypophosphatemic rickets with reduced expression of sodium phosphate cotransporter type IIa. Biochem Biophys Res Commun. 2004;314(2):409-14.

7. Shimada T, Hasegawa H, Yamazaki Y, Muto T, Hino R, Takeuchi Y, et al. FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. J Bone Miner Res. 2004;19(3):429-35.

8. Ben-Dov IZ, Galitzer H, Lavi-Moshayoff V, Goetz R, M K-o, Mohammadi M, et al. The parathyroid is a target organ for FGF23 in rats. J Clin Invest. 2007;117(12):4003-8.

9. Burnett SM, Gunawardene SC, Bringhurst FR, Juppner H, Lee H, Finkelstein JS. Regulation of C-terminal and intact FGF-23 by dietary phosphate in men and women. J Bone Miner Res. 2006;21(8):1187-96.

10. Perwad F, Azam N, Zhang MY, Yamashita T, Tenenhouse HS, Portale AA. Dietary and serum phosphorus regulate fibroblast growth factor 23 expression and 1,25-dihydroxyvitamin D metabolism in mice. Endocrinology. 2005;146(12):5358-64.

11. Vervloet MG, van Ittersum FJ, Buttler RM, Heijboer AC, Blankenstein MA, ter Wee PM. Effects of dietary phosphate and calcium intake on fibroblast growth factor-23. Clin J Am Soc Nephrol. 2011;6(2):383-9.

12. Evenepoel P, Meijers B, Viaene L, Bammens B, Claes K, Kuypers D, et al. Fibroblast growth factor-23 in early chronic kidney disease: additional support in favor of a phosphate-centric paradigm for the pathogenesis of secondary hyperparathyroidism. Clin J Am Soc Nephrol. 2010;5(7):1268-76.

13. Isakova T, Wahl P, Vargas GS, Gutierrez OM, Scialla J, Xie H, et al. Fibroblast growth factor 23 is elevated before parathyroid hormone and phosphate in chronic kidney disease. Kidney Int. 2011;79(12):1370-8.

14. Wolf M. Update on fibroblast growth factor 23 in chronic kidney disease. Kidney Int. 2012;82(7):737-47.

15. Shimada T, Urakawa I, Isakova T, Yamazaki Y, Epstein M, Wesseling-Perry K, et al. Circulating fibroblast growth factor 23 in patients with end-stage renal disease treated by peritoneal dialysis is intact and biologically active. J Clin Endocrinol Metab. 2010;95(2):578-85.

16. Yamazaki Y, Okazaki R, Shibata M, Hasegawa Y, Satoh K, Tajima T, et al. Increased circulatory level of biologically active full-length FGF-23 in patients with hypophosphatemic rickets/osteomalacia. J Clin Endocrinol Metab. 2002;87(11):4957-60.

17. Jonsson KB, Zahradnik R, Larsson T, White KE, Sugimoto T, Imanishi Y, et al. Fibroblast growth factor 23 in oncogenic osteomalacia and X-linked hypophosphatemia. N Engl J Med. 2003;348(17):1656-63.

18. Wolf M, White KE. Coupling fibroblast growth factor 23 production and cleavage: iron deficiency, rickets, and kidney disease. Current opinion in nephrology and hypertension. 2014;23(4):411-9.

19. Hu MC, Shi M, Zhang J, Pastor J, Nakatani T, Lanske B, et al. Klotho: a novel phosphaturic substance acting as an autocrine enzyme in the renal proximal tubule. FASEB J. 2010;24(9):3438-50.

20. Pavik I, Jaeger P, Ebner L, Wagner CA, Petzold K, Spichtig D, et al. Secreted Klotho and FGF23 in chronic kidney disease Stage 1 to 5: a sequence suggested from a cross-sectional study. Nephrol Dial Transplant. 2013;28(2):352-9.

21. Ohyama Y, Kurabayashi M, Masuda H, Nakamura T, Aihara Y, Kaname T, et al. Molecular cloning of rat klotho cDNA: markedly decreased expression of klotho by acute inflammatory stress. Biochem Biophys Res Commun. 1998;251(3):920-5.

22. Kim HR, Nam BY, Kim DW, Kang MW, Han JH, Lee MJ, et al. Circulating alpha-klotho levels in CKD and relationship to progression. Am J Kidney Dis. 2013;61(6):899-909.

23. Semba RD, Cappola AR, Sun K, Bandinelli S, Dalal M, Crasto C, et al. Plasma klotho and cardiovascular disease in adults. J Am Geriatr Soc. 2011;59(9):1596-601.

24. Lim K, Lu TS, Molostvov G, Lee C, Lam FT, Zehnder D, et al. Vascular Klotho deficiency potentiates the development of human artery calcification and mediates resistance to fibroblast growth factor 23. Circulation. 2012;125(18):2243-55.

25. Semba RD, Cappola AR, Sun K, Bandinelli S, Dalal M, Crasto C, et al. Plasma klotho and mortality risk in older community-dwelling adults. J Gerontol A Biol Sci Med Sci. 2011;66(7):794-800.

26. Hu MC, Shi M, Zhang J, Quinones H, Griffith C, Kuro-o M, et al. Klotho deficiency causes vascular calcification in chronic kidney disease. Journal of the American Society of Nephrology : JASN. 2011;22(1):124-36.

27. Isakova T, Xie H, Yang W, Xie D, Anderson AH, Scialla J, et al. Fibroblast growth factor 23 and risks of mortality and end-stage renal disease in patients with chronic kidney disease. JAMA. 2011;305(23):2432-9.

28. Seiler S, Reichart B, Roth D, Seibert E, Fliser D, Heine GH. FGF-23 and future cardiovascular events in patients with chronic kidney disease before initiation of dialysis treatment. Nephrol Dial Transplant. 2010;25(12):3983-9.

29. Kendrick J, Cheung AK, Kaufman JS, Greene T, Roberts WL, Smits G, et al. FGF-23 associates with death, cardiovascular events, and initiation of chronic dialysis. J Am Soc Nephrol. 2011;22(10):1913-22.

30. Gutierrez OM, Wolf M, Taylor EN. Fibroblast growth factor 23, cardiovascular disease risk factors, and phosphorus intake in the health professionals follow-up study. Clin J Am Soc Nephrol. 2011;6(12):2871-8.

31. Faul C, Amaral AP, Oskouei B, Hu MC, Sloan A, Isakova T, et al. FGF23 induces left ventricular hypertrophy. J Clin Invest. 2011;121(11):4393-408.

32. Baia LC, Humalda JK, Vervloet MG, Navis G, Bakker SJ, de Borst MH. Fibroblast growth factor 23 and cardiovascular mortality after kidney transplantation. Clin J Am Soc Nephrol. 2013;8(11):1968-78.

33. Qin Z, Liu X, Song M, Zhou Q, Yu J, Zhou B, et al. Fibroblast growth factor 23 as a predictor of cardiovascular and all-cause mortality in prospective studies. Atherosclerosis. 2017;261:1-11.

34. Goetz R, Nakada Y, Hu MC, Kurosu H, Wang L, Nakatani T, et al. Isolated C-terminal tail of FGF23 alleviates hypophosphatemia by inhibiting FGF23-FGFR-Klotho complex formation. Proc Natl Acad Sci U S A. 2010;107(1):407-12.

35. Rygasiewicz K, Hryszko T, Siemiatkowski A, Brzosko S, Rydzewska-Rosolowska A, Naumnik B. C-terminal and intact FGF23 in critical illness and their associations with acute kidney injury and in-hospital mortality. Cytokine. 2018;103:15-9.

36. Eisenga MF, van LM, Leaf DE, Nolte IM, Navis G, Bakker SJL, et al. C-Terminal Fibroblast Growth Factor 23, Iron Deficiency, and Mortality in Renal Transplant Recipients. J Am Soc Nephrol. 2017;28(12):3639-46.

37. Kanbay M, Vervloet M, Cozzolino M, Siriopol D, Covic A, Goldsmith D, et al. Novel Faces of Fibroblast Growth Factor 23 (FGF23): Iron Deficiency, Inflammation, Insulin Resistance, Left Ventricular Hypertrophy, Proteinuria and Acute Kidney Injury. Calcif Tissue Int. 2017;100(3):217-28.

38. Coe LM, Madathil SV, Casu C, Lanske B, Rivella S, Sitara D. FGF-23 is a negative regulator of prenatal and postnatal erythropoiesis. J Biol Chem. 2014;289(14):9795-810.

39. David V, Martin A, Isakova T, Spaulding C, Qi L, Ramirez V, et al. Inflammation and functional iron deficiency regulate fibroblast growth factor 23 production. Kidney Int. 2016;89(1):135-46.

40. Wyatt CM, Drueke TB. HIF stabilization by prolyl hydroxylase inhibitors for the treatment of anemia in chronic kidney disease. Kidney Int. 2016;90(5):923-5.

41. Mehta R, Cai X, Hodakowski A, Lee J, Leonard M, Ricardo A, et al. Fibroblast Growth Factor 23 and Anemia in the Chronic Renal Insufficiency Cohort Study. Clin J Am Soc Nephrol. 2017;12(11):1795-803.

42. Agoro R, Montagna A, Goetz R, Aligbe O, Singh G, Coe LM, et al. Inhibition of fibroblast growth factor 23 (FGF23) signaling rescues renal anemia. Faseb j. 2018;32(7):3752-64.

43. Patel NM, Gutierrez OM, Andress DL, Coyne DW, Levin A, Wolf M. Vitamin D deficiency and anemia in early chronic kidney disease. Kidney Int. 2010;77(8):715-20.

44. de Borst MH, Vervloet MG, ter Wee PM, Navis G. Cross talk between the renin-angiotensin-aldosterone system and vitamin D-FGF-23-klotho in chronic kidney disease. J Am Soc Nephrol. 2011;22(9):1603-9.

45. Humalda JK, Lambers Heerspink HJ, Kwakernaak AJ, Slagman MC, Waanders F, Vervloet MG, et al. Fibroblast growth factor 23 and the antiproteinuric response to dietary sodium restriction during renin-angiotensin-aldosterone system blockade. American journal of kidney diseases : the official journal of the National Kidney Foundation. 2015;65(2):259-66.

46. Ter Maaten JM, Voors AA, Damman K, van der Meer P, Anker SD, Cleland JG, et al. Fibroblast growth factor 23 is related to profiles indicating volume overload, poor therapy optimization and prognosis in patients with new-onset and worsening heart failure. International journal of cardiology. 2018;253:84-90.

47. Fishbane S, Pollack S, Feldman HI, Joffe MM. Iron indices in chronic kidney disease in the National Health and Nutritional Examination Survey 1988-2004. Clinical journal of the American Society of Nephrology : CJASN. 2009;4(1):57-61.

48. Ramey G, Deschemin JC, Durel B, Canonne-Hergaux F, Nicolas G, Vaulont S. Hepcidin targets ferroportin for degradation in hepatocytes. Haematologica. 2010;95(3):501-4.

49. Ashby DR, Gale DP, Busbridge M, Murphy KG, Duncan ND, Cairns TD, et al. Plasma hepcidin levels are elevated but responsive to erythropoietin therapy in renal disease. Kidney Int. 2009;75(9):976-81.

50. Weiss G, Theurl I, Eder S, Koppelstaetter C, Kurz K, Sonnweber T, et al. Serum hepcidin concentration in chronic haemodialysis patients: associations and effects of dialysis, iron and erythropoietin therapy. Eur J Clin Invest. 2009;39(10):883-90.

51. Tsuchiya K, Nitta K. Hepcidin is a potential regulator of iron status in chronic kidney disease. Ther Apher Dial. 2013;17(1):1-8.

52. Lippi G, Plebani M. Red blood cell distribution width (RDW) and human pathology. One size fits all. Clinical chemistry and laboratory medicine. 2014;52(9):1247-9.

53. Felker GM, Allen LA, Pocock SJ, Shaw LK, McMurray JJ, Pfeffer MA, et al. Red cell distribution width as a novel prognostic marker in heart failure: data from the CHARM Program and the Duke Databank. J Am Coll Cardiol. 2007;50(1):40-7.

54. Forhecz Z, Gombos T, Borgulya G, Pozsonyi Z, Prohaszka Z, Janoskuti L. Red cell distribution width in heart failure: prediction of clinical events and relationship with markers of ineffective erythropoiesis, inflammation, renal function, and nutritional state. Am Heart J. 2009;158(4):659-66.

55. Pascual-Figal DA, Bonaque JC, Redondo B, Caro C, Manzano-Fernandez S, Sanchez-Mas J, et al. Red blood cell distribution width predicts long-term outcome regardless of anaemia status in acute heart failure patients. Eur J Heart Fail. 2009;11(9):840-6.

56. Allen LA, Felker GM, Mehra MR, Chiong JR, Dunlap SH, Ghali JK, et al. Validation and potential mechanisms of red cell distribution width as a prognostic marker in heart failure. J Card Fail. 2010;16(3):230-8.

57. Tonelli M, Sacks F, Arnold M, Moye L, Davis B, Pfeffer M. Relation Between Red Blood Cell Distribution Width and Cardiovascular Event Rate in People With Coronary Disease. Circulation. 2008;117(2):163-8.

58. Oh HJ, Park JT, Kim JK, Yoo DE, Kim SJ, Han SH, et al. Red blood cell distribution width is an independent predictor of mortality in acute kidney injury patients treated with continuous renal replacement therapy. Nephrol Dial Transplant. 2012;27(2):589-94.

59. Vashistha T, Streja E, Molnar MZ, Rhee CM, Moradi H, Soohoo M, et al. Red Cell Distribution Width and Mortality in Hemodialysis Patients. Am J Kidney Dis. 2016;68(1):110-21.

60. Patel KV, Ferrucci L, Ershler WB, Longo DL, Guralnik JM. Red blood cell distribution width and the risk of death in middle-aged and older adults. Arch Intern Med. 2009;169(5):515-23.

61. Perlstein TS, Weuve J, Pfeffer MA, Beckman JA. Red blood cell distribution width and mortality risk in a community-based prospective cohort. Arch Intern Med. 2009;169(6):588-94.

62. Emans ME, van der Putten K, van Rooijen KL, Kraaijenhagen RJ, Swinkels D, van Solinge WW, et al. Determinants of red cell distribution width (RDW) in cardiorenal patients: RDW is not related to erythropoietin resistance. J Card Fail. 2011;17(8):626-33.

63. Anker SD, Comin CJ, Filippatos G, Willenheimer R, Dickstein K, Drexler H, et al. Ferric carboxymaltose in patients with heart failure and iron deficiency. N Engl J Med. 2009;361(25):2436-48.

64. Jankowska EA, Kasztura M, Sokolski M, Bronisz M, Nawrocka S, Oleskowska-Florek W, et al. Iron deficiency defined as depleted iron stores accompanied by unmet cellular iron requirements identifies patients at the highest risk of death after an episode of acute heart failure. European heart journal. 2014;35(36):2468-76.

65. Ponikowski P, Voors AA, Anker SD, Bueno H, Cleland JG, Coats AJ, et al. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC). Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. European journal of heart failure. 2016;18(8):891-975.

66. Al-Ahmad A, Rand WM, Manjunath G, Konstam MA, Salem DN, Levey AS, et al. Reduced kidney function and anemia as risk factors for mortality in patients with left ventricular dysfunction. J Am Coll Cardiol. 2001;38(4):955-62.

67. Xu D, Murakoshi N, Sairenchi T, Irie F, Igarashi M, Nogami A, et al. Anemia and reduced kidney function as risk factors for new onset of atrial fibrillation (from the Ibaraki prefectural health study). Am J Cardiol. 2015;115(3):328-33.

68. Levin A, Thompson CR, Ethier J, Carlisle EJ, Tobe S, Mendelssohn D, et al. Left ventricular mass index increase in early renal disease: impact of decline in hemoglobin. Am J Kidney Dis. 1999;34(1):125-34.

69. Foley RN, Parfrey PS, Harnett JD, Kent GM, Murray DC, Barre PE. The impact of anemia on cardiomyopathy, morbidity, and and mortality in end-stage renal disease. Am J Kidney Dis. 1996;28(1):53-61.

70. Eriksson D, Goldsmith D, Teitsson S, Jackson J, van NF. Cross-sectional survey in CKD patients across Europe describing the association between quality of life and anaemia. BMC Nephrol. 2016;17(1):97.

71. Alexander M, Kewalramani R, Agodoa I, Globe D. Association of anemia correction with health related quality of life in patients not on dialysis. Curr Med Res Opin. 2007;23(12):2997-3008.

72. Feldman HI, Santanna J, Guo W, Furst H, Franklin E, Joffe M, et al. Iron administration and clinical outcomes in hemodialysis patients. Journal of the American Society of Nephrology : JASN. 2002;13(3):734-44.

73. Feldman HI, Joffe M, Robinson B, Knauss J, Cizman B, Guo W, et al. Administration of parenteral iron and mortality among hemodialysis patients. Journal of the American Society of Nephrology : JASN. 2004;15(6):1623-32.

74. Kuo KL, Hung SC, Liu JS, Chang YK, Hsu CC, Tarng DC. Iron supplementation associates with low mortality in pre-dialyzed advanced chronic kidney disease patients receiving erythropoiesis-stimulating agents: a nationwide database analysis. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2015;30(9):1518-25.

75. White KE, Carn G, Lorenz-Depiereux B, Benet-Pages A, Strom TM, Econs MJ. Autosomal-dominant hypophosphatemic rickets (ADHR) mutations stabilize FGF-23. Kidney Int. 2001;60(6):2079-86.

76. Shimada T, Muto T, Urakawa I, Yoneya T, Yamazaki Y, Okawa K, et al. Mutant FGF-23 responsible for autosomal dominant hypophosphatemic rickets is resistant to proteolytic cleavage and causes hypophosphatemia in vivo. Endocrinology. 2002;143(8):3179-82.

77. Bianchine JW, Stambler AA, Harrison HE. Familial hypophosphatemic rickets showing autosomal dominant inheritance. Birth Defects Orig Artic Ser. 1971;7(6):287-95.

78. Econs MJ, McEnery PT. Autosomal dominant hypophosphatemic rickets/osteomalacia: clinical characterization of a novel renal phosphate-wasting disorder. J Clin Endocrinol Metab. 1997;82(2):674-81.

79. Farrow EG, Yu X, Summers LJ, Davis SI, Fleet JC, Allen MR, et al. Iron deficiency drives an autosomal dominant hypophosphatemic rickets (ADHR) phenotype in fibroblast growth factor-23 (Fgf23) knock-in mice. Proc Natl Acad Sci U S A. 2011;108(46):E1146-E55.

80. Clinkenbeard EL, Farrow EG, Summers LJ, Cass TA, Roberts JL, Bayt CA, et al. Neonatal iron deficiency causes abnormal phosphate metabolism by elevating FGF23 in normal and ADHR mice. J Bone Miner Res. 2014;29(2):361-9.

81. Wolf M, Koch TA, Bregman DB. Effects of iron deficiency anemia and its treatment on fibroblast growth factor 23 and phosphate homeostasis in women. J Bone Miner Res. 2013.

82. Gravesen E, Hofman-Bang J, Mace ML, Lewin E, Olgaard K. High dose intravenous iron, mineral homeostasis and intact FGF23 in normal and uremic rats. BMC Nephrol. 2013;14:281.

83. Prats M, Font R, Garcia C, Cabre C, Jariod M, Vea AM. Effect of ferric carboxymaltose on serum phosphate and C-terminal FGF23 levels in non-dialysis chronic kidney disease patients: post-hoc analysis of a prospective study. BMC Nephrol. 2013;14:167.

84. Hryszko T, Rydzewska-Rosolowska A, Brzosko S, Koc-Zorawska E, Mysliwiec M. Low molecular weight iron dextran increases fibroblast growth factor-23 concentration, together with parathyroid hormone decrease in hemodialyzed patients. Ther Apher Dial. 2012;16(2):146-51.

85. Takeda Y, Komaba H, Goto S, Fujii H, Umezu M, Hasegawa H, et al. Effect of intravenous saccharated ferric oxide on serum FGF23 and mineral metabolism in hemodialysis patients. Am J Nephrol. 2011;33(5):421-6.

86. Deger SM, Erten Y, Pasaoglu OT, Derici UB, Reis KA, Onec K, et al. The effects of iron on FGF23-mediated Ca-P metabolism in CKD patients. Clin Exp Nephrol. 2013;17(3):416-23.

87. Holick MF. Vitamin D deficiency. N Engl J Med. 2007;357(3):266-81.

88. Icardi A, Paoletti E, De NL, Mazzaferro S, Russo R, Cozzolino M. Renal anaemia and EPO hyporesponsiveness associated with vitamin D deficiency: the potential role of inflammation. Nephrol Dial Transplant. 2013;28(7):1672-9.

89. Gouni-Berthold I, Krone W, Berthold HK. Vitamin D and cardiovascular disease. Curr Vasc Pharmacol. 2009;7(3):414-22.

90. Zhou C, Lu F, Cao K, Xu D, Goltzman D, Miao D. Calcium-independent and 1,25(OH)2D3-dependent regulation of the renin-angiotensin system in 1alpha-hydroxylase knockout mice. Kidney Int. 2008;74(2):170-9.

91. Li YC, Kong J, Wei M, Chen ZF, Liu SQ, Cao LP. 1,25-Dihydroxyvitamin D(3) is a negative endocrine regulator of the renin-angiotensin system. The Journal of clinical investigation. 2002;110(2):229-38.

92. Nigwekar SU, Bhan I, Thadhani R. Ergocalciferol and cholecalciferol in CKD. Am J Kidney Dis. 2012;60(1):139-56.

93. Kendrick J, Cheung AK, Kaufman JS, Greene T, Roberts WL, Smits G, et al. Associations of plasma 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D concentrations with death and progression to maintenance dialysis in patients with advanced kidney disease. Am J Kidney Dis. 2012;60(4):567-75.

94. Thadhani R, Appelbaum E, Pritchett Y, Chang Y, Wenger J, Tamez H, et al. Vitamin D therapy and cardiac structure and function in patients with chronic kidney disease: the PRIMO randomized controlled trial. Jama. 2012;307(7):674-84.

95. Wang AY, Fang F, Chan J, Wen YY, Qing S, Chan IH, et al. Effect of paricalcitol on left ventricular mass and function in CKD--the OPERA trial. Journal of the American Society of Nephrology : JASN. 2014;25(1):175-86.

96. Lu RJ, Zhu SM, Tang FL, Zhu XS, Fan ZD, Wang GL, et al. Effects of vitamin D or its analogues on the mortality of patients with chronic kidney disease: an updated systematic review and meta-analysis. Eur J Clin Nutr. 2017;71(6):683-93.

97. Lucisano S, Di ME, Montalto G, Cernaro V, Buemi M, Santoro D. Vitamin D and anemia. J Ren Nutr. 2014;24(1):61-2.

98. Albitar S, Genin R, Fen-Chong M, Serveaux MO, Schohn D, Chuet C. High-dose alfacalcidol improves anaemia in patients on haemodialysis. Nephrol Dial Transplant. 1997;12(3):514-8.

99. Saab G, Young DO, Gincherman Y, Giles K, Norwood K, Coyne DW. Prevalence of vitamin D deficiency and the safety and effectiveness of monthly ergocalciferol in hemodialysis patients. Nephron Clin Pract. 2007;105(3):c132-c8.

100. Bacchetta J, Zaritsky JJ, Sea JL, Chun RF, Lisse TS, Zavala K, et al. Suppression of iron-regulatory hepcidin by vitamin D. J Am Soc Nephrol. 2014;25(3):564-72.

101. Brancaccio D, Cozzolino M, Gallieni M. Hyperparathyroidism and anemia in uremic subjects: a combined therapeutic approach. J Am Soc Nephrol. 2004;15 Suppl 1:S21-S4.

102. Moorthi RN, Kandula P, Moe SM. Optimal vitamin D, calcitriol, and vitamin D analog replacement in chronic kidney disease: to D or not to D: that is the question. Curr Opin Nephrol Hypertens. 2011;20(4):354-9.

103. Ruggenenti P, Perna A, Remuzzi G. Retarding progression of chronic renal disease: the neglected issue of residual proteinuria. Kidney Int. 2003;63(6):2254-61.

104. Holtkamp FA, de ZD, de Graeff PA, Laverman GD, Berl T, Remuzzi G, et al. Albuminuria and blood pressure, independent targets for cardioprotective therapy in patients with diabetes and nephropathy: a post hoc analysis of the combined RENAAL and IDNT trials. Eur Heart J. 2011;32(12):1493-9.

105. Wolf G, Ritz E. Combination therapy with ACE inhibitors and angiotensin II receptor blockers to halt progression of chronic renal disease: pathophysiology and indications. Kidney Int. 2005;67(3):799-812.

106. Yusuf S, Teo KK, Pogue J, Dyal L, Copland I, Schumacher H, et al. Telmisartan, ramipril, or both in patients at high risk for vascular events. N Engl J Med. 2008;358(15):1547-59.

107. Burgess ED, Hawkins RG, Watanabe M. Interaction of 1,25-dihydroxyvitamin D and plasma renin activity in high renin essential hypertension. Am J Hypertens. 1990;3(12 Pt 1):903-5.

108. Resnick LM, Muller FB, Laragh JH. Calcium-regulating hormones in essential hypertension. Relation to plasma renin activity and sodium metabolism. Ann Intern Med. 1986;105(5):649-54.

109. de ZD, Agarwal R, Amdahl M, Audhya P, Coyne D, Garimella T, et al. Selective vitamin D receptor activation with paricalcitol for reduction of albuminuria in patients with type 2 diabetes (VITAL study): a randomised controlled trial. Lancet. 2010;376(9752):1543-51.

110. de Borst MH, Hajhosseiny R, Tamez H, Wenger J, Thadhani R, Goldsmith DJ. Active vitamin D treatment for reduction of residual proteinuria: a systematic review. Journal of the American Society of Nephrology : JASN. 2013;24(11):1863-71.

111. Abbate M, Zoja C, Remuzzi G. How does proteinuria cause progressive renal damage? J Am Soc Nephrol. 2006;17(11):2974-84.

112. Caruso-Neves C, Pinheiro AA, Cai H, Souza-Menezes J, Guggino WB. PKB and megalin determine the survival or death of renal proximal tubule cells. Proc Natl Acad Sci U S A. 2006;103(49):18810-5.

113. Breyer JA, Bain RP, Evans JK, Nahman NS, Jr., Lewis EJ, Cooper M, et al. Predictors of the progression of renal insufficiency in patients with insulin-dependent diabetes and overt diabetic nephropathy. The Collaborative Study Group. Kidney Int. 1996;50(5):1651-8.

114. Peterson JC, Adler S, Burkart JM, Greene T, Hebert LA, Hunsicker LG, et al. Blood pressure control, proteinuria, and the progression of renal disease. The Modification of Diet in Renal Disease Study. Ann Intern Med. 1995;123(10):754-62.

115. Matsushita K, van d, V, Astor BC, Woodward M, Levey AS, de Jong PE, et al. Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis. Lancet. 2010;375(9731):2073-81.

116. Brenner BM, Cooper ME, de ZD, Keane WF, Mitch WE, Parving HH, et al. Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. N Engl J Med. 2001;345(12):861-9.

117. Yusuf S, Sleight P, Pogue J, Bosch J, Davies R, Dagenais G. Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. N Engl J Med. 2000;342(3):145-53.

118. Maschio G, Alberti D, Janin G, Locatelli F, Mann JF, Motolese M, et al. Effect of the angiotensin-converting-enzyme inhibitor benazepril on the progression of chronic renal insufficiency. The Angiotensin-Converting-Enzyme Inhibition in Progressive Renal Insufficiency Study Group. N Engl J Med. 1996;334(15):939-45.

119. Matsui I, Hamano T, Tomida K, Inoue K, Takabatake Y, Nagasawa Y, et al. Active vitamin D and its analogue, 22-oxacalcitriol, ameliorate puromycin aminonucleoside-induced nephrosis in rats. Nephrol Dial Transplant. 2009;24(8):2354-61.

120. Safadi FF, Thornton P, Magiera H, Hollis BW, Gentile M, Haddad JG, et al. Osteopathy and resistance to vitamin D toxicity in mice null for vitamin D binding protein. J Clin Invest. 1999;103(2):239-51.

121. Zoccali C, Ruggenenti P, Perna A, Leonardis D, Tripepi R, Tripepi G, et al. Phosphate may promote CKD progression and attenuate renoprotective effect of ACE inhibition. Journal of the American Society of Nephrology : JASN. 2011;22(10):1923-30.

122. Vervloet MG, van Zuilen AD, Heijboer AC, ter Wee PM, Bots ML, Blankestijn PJ, et al. Fibroblast growth factor 23 is associated with proteinuria and smoking in chronic kidney disease: an analysis of the MASTERPLAN cohort. BMC nephrology. 2012;13:20.

123. de Seigneux S, Courbebaisse M, Rutkowski JM, Wilhelm-Bals A, Metzger M, Khodo SN, et al. Proteinuria Increases Plasma Phosphate by Altering Its Tubular Handling. Journal of the American Society of Nephrology : JASN. 2015;26(7):1608-18.

Chapter 2

RELATION BETWEEN RED CELL DISTRIBUTION WIDTH AND FIBROBLAST GROWTH FACTOR 23 CLEAVING IN PATIENTS WITH CHRONIC KIDNEY DISEASE AND HEART FAILURE

G. Fenna van Breda[1], Mireille E. Emans[2], Karien van der Putten[3], Branko Braam[4], Frans J. van Ittersum[1], Rob J. Kraaijenhagen[5], Martin H. de Borst[6], Marc Vervloet[1], Carlo A.J.M. Gaillard[6]

1. Department of Nephrology and ICaR-VU, VUMC, Amsterdam, the Netherlands
2. Department of Cardiology, Ikazia Hospital, Rotterdam, the Netherlands
3. Department of Internal Medicine, TerGooi Hospital, Hilversum, the Netherlands
4. Department of Medicine, division of Nephrology and Immunology, University of Alberta, Edmonton, Canada
5. Department of Clinical Chemistry, Meander Medical Center, Amersfoort, the Netherlands
6. Department of Nephrology, UMCG, Groningen, the Netherlands

Plos One 2015 Jun 16; 10(6)):e0128994. doi: 10.1371/journal.pone.0128994

Abstract

Objective: In chronic kidney disease (CKD), both anemia and deregulated phosphate metabolism are common and predictive of adverse outcome. Previous studies suggest that iron status influences phosphate metabolism by modulating proteolytic cleavage of FGF23 into C-terminal fragments. Red cell distribution width (RDW) was recently identified as a strong prognostic determinant for cardiovascular morbidity and mortality, independently of iron status. We assessed whether RDW is associated with FGF23 cleaving in CKD patients with heart failure.

Materials and methods: The associations between RDW and either intact FGF23 (iFGF23), C-terminal FGF23 (cFGF23, reflecting iFGF23 and C-terminal fragments together) and the iFGF23/cFGF23 ratio were analyzed in 52 patients with CKD (eGFR 34,9 ± 13.9 ml/min/1.73m^2) and chronic heart failure (CHF). Associations between RDW and FGF23 forms were studied by linear regression analysis adjusted for parameters of renal function, iron metabolism, phosphate metabolism and inflammation.

Results: Median cFGF23 levels were 197.5 [110-408.5] RU/ml, median iFGF23 levels were 107.3 [65.1-162.2] pg/ml and median FGF23 ratio was 0.80 [0.37-0.86]. Mean RDW was 14.1 ± 1.2%. cFGF23 and RDW were associated (β= 1.63x10^{-3}, P <0.001), whereas iFGF23 and RDW were not (β= -1.38x10$^{-3,}$ P= 0.336). The iFGF23/cFGF23 ratio was inversely associated with RDW. The difference between cFGF23 and iFGF23 (cFGF23- iFGF23) was positively associated with RDW (β= 1.74x10^{-3}, P< 0.001). The association between cFGF23 and RDW persisted upon multivariable linear regression analysis, adjusted for parameters of renal function, phosphate metabolism, iron metabolism and inflammation (β= 0.97x10^{-3}, P= 0.047).

Conclusion: RDW is associated with cFGF23 but not with iFGF23 levels in patients with CKD and CHF. This suggests a connection between RDW and FGF23 catabolism, independent of iron status and inflammation. Future studies are needed to unravel underlying mechanisms and whether these pertain to the link between RDW and outcome.

Keywords: Chronic kidney failure, fibroblast growth factor 23, red cell distribution width, chronic heart failure

Introduction

The simultaneous occurrence of chronic heart failure (CHF) and chronic kidney disease (CKD), known as the cardiorenal syndrome (CRS), is accompanied by high morbidity and mortality (1,2). Traditional risk factors only partly explain this high risk (3), suggesting that additional pathophysiological mechanisms are involved. Several novel risk factors have been implicated in the elevated cardiovascular risk in CKD. Prominent non-traditional risk factors include red cell related measures such as anemia, iron status and red cell distribution width (RDW) (4), and markers of mineral metabolism, especially fibroblast growth factor 23 (FGF23) (5). Interestingly, recent studies suggest a mechanistic link between these two systems (6–8).

FGF23 is a bone derived phosphaturic hormone that plays an important role in systemic phosphate homeostasis and vitamin D metabolism. Several observational studies consistently demonstrate independent associations between FGF23 and accelerated CKD progression (9), left ventricular hypertrophy in dialysis and predialysis patients (10), and increased mortality risk in CKD and hemodialysis patients and kidney transplant recipients (10–14). Recently, it was shown that iron status influences FGF23 catabolism in mice with autosomal dominant hypophosphatemic rickets (6). Similarly, in female patients with iron deficient anemia markedly elevated C-terminal FGF23 (cFGF23) levels but not intact FGF23 (iFGF23) levels were found (7). Importantly, intravenous iron administration markedly reduced cFGF23 levels, providing another clue that iron status influences FGF23 cleaving. The current hypothesis is that, in healthy individuals, iron deficiency stimulates FGF23 production whereby osteocytes couple increased production of FGF23 with increased cleavage to cFGF23 to maintain normal circulating levels of iFGF23, which is the biologically intact hormone (15). However, it is unknown whether this finding holds for CKD, a disease characterized by disturbed iron metabolism, high FGF23 levels and increased risk for cardiovascular complications.

Red cell distribution width (RDW) is a measure of the variation of red blood cell volume, defined as the standard deviation of erythrocyte size divided by the mean corpuscular volume. RDW is a robust marker of adverse clinical outcomes in patients with chronic and acute heart failure (16–19), coronary artery disease (20), acute kidney injury (AKI) (21) and even in the community (22–24). The pathophysiological mechanism responsible for the association between RDW and adverse outcomes remains to be resolved, but could be related to disturbed iron metabolism or inflammation (19,25). Because both FGF23 and RDW are independently associated with poor outcome measures, and both seem to be affected by iron, it is interesting to investigate whether a relation exists between FGF23 and RDW.

We hypothesized that a higher RDW is associated with more FGF23 cleavage, providing a common pathway in which both markers lead to adverse outcomes. Therefore, we examined the relationship between RDW and both intact and C-terminal FGF23 as well as the ratio between the two, and the difference between cFGF23 and iFGF23, in a cohort of patients with chronic kidney disease and chronic heart failure. Analyses were adjusted for markers of renal function, iron status and inflammation.

Methods

Subjects

For the current study we performed a post hoc cross-sectional analysis of baseline data from patients enrolled in the EPOCARES study (The Mechanisms of Erythropoetin Action in the CardioRenal Syndrome, ClinicalTrials.gov NCT 00356733). The study design of the EPOCARES study has been published previously (26). The study is being carried out in compliance with the Helsinki Declaration, and the protocol has been approved at each participating center by its internal review board. In short, the EPOCARES study is an open-label, prospective, randomized trial in which patients with CHF, CKD (glomerular filtration rate 20-70 ml/min) and mild anemia (hemoglobin 10.3-12.6 g/dL in men, and 10.3-11,9 g/dL in women) were included to test the erythropoietic and non-erythropoietic responses to low-dose ESA treatment. Patients with active systemic disease as a cause of CHF or CKD were excluded. Other exclusion criteria were ESA therapy in the previous 6 months, bleeding, chronic inflammatory disease or malignancy. In all patients, standard treatment was started, comprising oral iron suppletion (ferrofumarate), calcium carbonate, aspirin when indicated and maximal tolerated dosages of a β-blocker, an angiotensin-converting enzyme (ACE) inhibitor or an angiotensin receptor blocker, according to CHF guidelines. Included patients were randomized into 3 groups: 1 group received a fixed dose of 50 IU/kg per week EPO to increase hemoglobin level to a maximum of 13.7 g/dL for men and 13.4 g/dL for women; another group was treated with 50 IU/kg per week EPO maintaining baseline hemoglobin levels for the first 6 months by phlebotomy. The control group received standard care without EPO. This translational study was designed primarily to discern hematopoietic from nonhematopoietic effects of erythropoietin (EPO) in cardiorenal patients. All baseline data were derived prior to randomization and initiation of EPO treatment.

The original study population of the EPOCARES study consisted of 62 patients. Five patients withdrew their informed consent and one patient was excluded because of malignancy diagnosed after inclusion. Baseline RDW data were missing for two patients and two outliers of FGF23 were excluded, since these values exceeded the third quartile by a magnitude greater than 1.5 (IQR).

Biochemical analysis

Biochemical measurements were performed at baseline and blood samples were drawn between 8 and 9 AM in supine position and stored immediately at -80 °C until analysis.

Levels of Hb, hematocrit, MCV and RDW were measured using a Sysmex XE-2100 hematology analyzer (Toa Medical, Kobe, Japan). Plasma interleukin-6 (IL-6) levels were measured in duplo using a commercially available ELISA kit (R&D Systems, Minneapolis, USA).

As a marker of iron stores (27) , ferritin was determined using a sandwich immunoassay on an Acces 2 immunoanalyzer within a Dx automated system from Beckman Coulter (Brea, CA). Function iron availability was determined by measuring transferrin saturation (TSAT) and was calculated from serum iron and transferrin estimates obtained with standard methods on a Beckman Coulter Dx. Renal function was estimated by means of MDRD. Reference values of all parameters are shown in table 1.

FGF23 was analyzed with two validated assays (28). The iFGF23 was determined in serum using a sandwich ELISA, (Kainos Laboratories, Tokio, Japan), the intra- and interassay CV's are <10% and <14%, respectively. The cFGF23 was assessed in EDTA-plasma using a sandwich enzyme-linked immunosorbent assay (ELISA) (Immutopics, San Clemente, CA, USA). The intra- and interassay CV's are <5% and <16%, respectively. The former assay detects only the full-length FGF23, while the latter assay additionally measures the c-terminal fragments of truncated FGF23. In order to estimate the amount of intact FGF23 in relation to the total amount of FGF23 (i.e. intact FGF23 + C-terminal FGF23 as measured by the C-terminal assay), we calculated the iFGF23/cFGF23 ratio. We also estimated the absolute amount of C-terminal fragments by calculating the difference between total FGF23 and iFGF23.

Statistical analysis

Continuous variables at baseline were summarized as the mean ± standard deviation (SD) if normally distributed or otherwise as medians with interquartile range (IQR). Skewed variables were transformed to natural logarithms after which normality was checked again. After checking model assumptions, univariate linear regression analyses were used to test the relationship between iFGF23, cFGF23 and the difference between cFGF23 and iFGF23 (cFGF23-iFGF23) with RDW. The ratio iFGF23/cFGF23 was divided in tertiles because of violation of linearity and we used two dummies to estimate the regression coefficient between ratio iFGF23/cFGF23 and RDW. FGF23 was used as independent variable and RDW as dependent variable. To test the relationship between FGF23 and TSAT, univariate regression analysis was performed with TSAT as independent

and FGF23 as dependent variable. Subsequently, four multivariable linear regression models were used to adjust for confounding of the primary analysis (RDW and cFGF23). A 10% change of the regression coefficient was considered to indicate relevant confounding. Model 1 adjusted for potential confounders of the relationship between cFGF23 and RDW derived from the literature (29–31): eGFR, PTH, phosphate, BMI and smoking. Model 2 adjusted for variables used in model 1 and in addition markers of iron metabolism (TSAT and ferritin). Model 3 adjusted for variables used in model 1 and in addition markers of inflammation (IL-6 and CRP). In the final model 4, a combination of previous models was used to adjust for all possible confounders. Age and sex were ruled out to be effect modifiers. For statistical analysis, the SPSS software package version 20 was used (SPSS, IBM, Chicago, IL, USA).

Results

Population characteristics

Demographics, baseline laboratory data and clinical characteristics of the 52 patients enrolled into this study are reported in Table 1.

Median age was 73 years (IQR 69-80) and 63.5% were male (Table 1). Of the population included in this study 11.5% were smokers and on average BMI was elevated (median 25.9, IQR 23.7-29.9). The mean RDW value was 14.1% ± 1.2, with a reference range of 10.4-13.0% and the mean eGFR 35 ± 14 ml/min/1.73m^2. Both iFGF23 (median 107.3 pg/ml, IQR 65.1-162.2) and cFGF23 levels (median 197.5 RU/ml, IQR 110-408.5) were increased. CRP levels were only slightly elevated, showing that the study involved chronic stable patients in a relatively low-inflammatory state. Ferritin levels and TSAT were low-normal.

Relation between FGF23 and TSAT

Univariate linear regression was performed to estimate the relation between FGF23 and TSAT. A statistically significant association was found between TSAT and cFGF23 (β= -12.35, P= 0.03), but not between TSAT and iFGF23 (β= -1.86, P= 0.31).

Table 1. Main clinical and biochemical characteristics of patients from the EPOCARES study at baseline

Characteristics*	All patients n=52	Reference values
Age (yrs)	73 [69-80]	
Male sex, n (%)	33 (63.5%)	
Smoking (%)	11.5%	
BMI kg/m²	25.9 [23.7-29.9]	
Diabetes Mellitus	36.5%	
Hypertension	78.8%	
Hemoglobin (g/dL)	11.8 ± 0.9	12.5 – 16.1 g/dL (f)
		13.7 – 17.0 g/dL (m)
Hematocrit (L/L)	0.35 ± 0.03	0.36 – 0.48 L/L (f)
		0.40 – 0.52 L/L (m)
MCV (/µm³)	90 ± 4	80 - 102
RDW (%)	14.1 ± 1.2	10.4-13.0 %
MDRD (ml/min/1.73m²)	35 ± 14	> 60 ml/min/1.73m²
NT-proBNP (pg/mL)	1387 [688-2370]	<738 pg/ml
Ferritin (ng/mL)	129 [75-179]	10 – 200 ug/l
Iron (µmol/L)	10 [8.8-14]	9 – 30 umol/l
TSAT (%)	20 [16.3-25]	< 45 %
CRP (mg/L)	5 [2-11.3]	0 – 10 mg/l
IL-6 (pg/mL)	3.27 [1.9-5]	< 10 pg/mL
iFGF23	107.3 [65.1-162.2]	20-50 pg/ml
cFGF23	197.5 [110-408.5]	< 125 RU/ml
iFGF23/cFGF23	0.803 [0.37-0.86]	
PTH	10.4 [6.7-15]	1.5 – 7 pmol/l
Phosphate	1.15 [1-1.2]	0.80 – 1.45 mmol/l

BMI= body mass index, MCV= mean corpuscular volume, RDW= red cell distribution width, MDRD= estimated glomerular filtration rate by modified diet in renal disease formula, NT-proBNP= N-terminal pro-brain natriuretic peptide, TSAT= transferrin saturation, CRP= C-reactive protein, IL-6= interleukin 6, iFGF23= intact fibroblast growth factor 23, cFGF23= C-terminal fibroblast growth factor 23, PTH= parathyreoid hormone. *values in mean ± standard deviation or median [interquartile range]

Relation between FGF23 and RDW

Univariate linear regression showed a statistically significant relationship between cFGF23 and RDW (β= 1.63 x 10^{-3}, P <0.001) in our population (Fig. 1A), but not between iFGF23 and RDW (β= -1.38 x 10^{-3}, P= 0.34, Fig. 1B). The difference between cFGF23 and iFGF23 (cFGF23-iFGF23, representing the amount of c-terminal FGF23 fragments) was positively correlated with RDW (β= 1.74x10^{-3}, P< 0.001, Fig. 1C). In order to comply with the conditions for linear regression, we divided the iFGF23/cFGF23 ratio (representing the fraction of intact, biologically active FGF23) into tertiles. Both the second and the third tertile of the iFGF23/cFGF23 ratio were associated with RDW (β= -0.947, P= 0.014 and β= -1.253 and P= 0.002). This might be explained by reduced iron availability, as iron availability is a determinant of both RDW as well as the iFGF23/cFGF23 ratio due to increased cleavage of iFGF23 into C-terminal fragments in conditions of reduced iron availability.

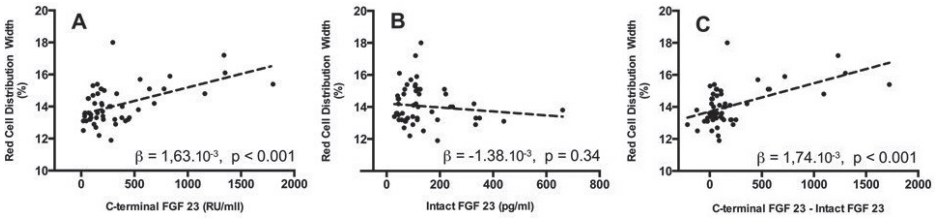


Fig 1. The relationship between baseline cFGF23 and RDW (A), baseline iFGF23 and RDW (B) and between cFGF23-iFGF23 and RDW (C).

To further analyze the relation of cFGF23 with RDW, we constructed several models to adjust for potential confounders (Table 2). Adjustment for eGFR, PTH, phosphate, BMI and smoking (model 1) did not modify the regression coefficient significantly between cFGF23 and RDW (β= 1.5 x 10^{-3}, P= 0.001). Further adjustment for indicators of iron deficiency, ferritin and TSAT (model 2), marginally attenuated the association (β= 1.34 x 10^{-3}, P= 0.003). Model 3, correcting for variables used in model 1 and for CRP and IL-6 as markers of inflammation, further attenuated the strength of the cFGF23-RDW association (β= 1.08x10^{-3}, P= 0.023). After adjusting for all mentioned variables (model 4), the association between cFGF23 and RDW remained statistically significant (β= 0.969x10^{-3}, P= 0.047).

In a sensitivity analysis, further adjusted for hypertension, diabetes mellitus, hemoglobin level and 25-hydroxyvitamin D did not influence this relation (data not shown).

Table 2. Multivariable linear regression for association between cFGF23 and RDW after adjustment for confounders

Y= RDW	Regressioncoefficient	p-value
Crude analysis		
cFGF23 RU/ml	1.63 x 10^{-3}	<0.001
Adjusted analysis 1*		
cFGF23 RU/ml	1.50 x 10^{-3}	0.01
Adjusted analysis 2*		
cFGF23 RU/ml	1.34 x 10^{-3}	0.003
Adjusted analysis 3*		
cFGF23 RU/ml	1.08 x 10^{-3}	0.023
Adjusted analysis 4*		
cFGF23 RU/ml	0.97 x 10^{-3}	0.047

1. Adjusted for eGFR, PTH, Phosphate, BMI and smoking
2. Adjusted for eGFR, PTH, phosphate, BMI, smoking, ferritin and TSAT
3. Adjusted for eGFR, PTH, phosphate, BMI, smoking, IL-6 and CRP
4. Adjusted for eGFR, PTH, phosphate, BMI, smoking, ferritin, TSAT, IL-6 and CRP

Discussion

The main finding of this study is the strong and robust association between cFGF23 levels and RDW in patients with CKD and CHF, which persisted after adjustment of several potential confounders. Interestingly, in contrast with the consistent association between cFGF23 and RDW, no association between iFGF23 and RDW was observed. Our results are in line with previous observations connecting red cell properties with phosphate homeostasis (6,7). As both red cell properties and calcium/phosphate homeostasis are important prognostic factors in CKD, detailed knowledge about their interaction could provide novel insights into the etiology of combined CHF and CKD and the subsequent deteriorated prognosis.

Currently, the underlying pathophysiological mechanisms linking FGF23 and RDW with outcome are unknown. Our data suggest an association between cFGF23 and RDW, raising the question whether there is an unknown factor that directly affects the risk of adverse outcome in combined CHF and CKD and whether this also affects both RDW and FGF23. Therefore, co-aggregation of changes in FGF23 and RDW caused by an established factor associated with renal function (i.e. potential confounding) should be ruled out. We adjusted for several potential confounders, known to influence FGF23 concentrations, based on the literature (eGFR, PTH, phosphate, smoking, and BMI) (29–31): this did not substantially mitigate the strength of the association between RDW and FGF23.

Recent data demonstrated that iron deficiency can increase cFGF23, possibly as a result of increased FGF23 cleaving (6,7). Wolf et al. demonstrated that iron deficiency stimulates FGF23 transcription whereby increased levels of iFGF23 are cleaved intracellularly into cFGF23 in healthy humans as such limiting its physiological effects on phosphate homeostasis. Therefore, iron metabolism may be a link between FGF23 and RDW in our patients with combined chronic heart and renal failure. Although we found a significant association between cFGF23 and TSAT, the association between cFGF23 and RDW was only marginally attenuated by TSAT and ferritin. This suggests that additional mechanisms could be involved. Of note, the study design of the EPOCARES study included oral iron supplementation in all groups.

In addition to iron metabolism, inflammation stands out as a potential mechanism explaining the association between FGF23 and RDW. Higher levels of FGF23 are independently associated with inflammation in patients with CKD (32) and high RDW values have been associated with plasma markers of inflammation in a large cohort of unselected adult outpatients (33) and in patients with heart failure (17). Indeed, our subsequent analysis showed that the strength of the

association between cFGF23 and RDW was attenuated, but not abolished, after adjusting for CRP and IL-6. Taken together, the association between cFGF23 and RDW may be partly explained by iron metabolism as well as by inflammation but remains statistically significant after adjustment for these factors, indicating that additional unknown factors may link cFGF23 and RDW.

Alternatively, cFGF23 may directly influence RDW. Although there is some debate, several studies demonstrate FGF23 effects on the vessel wall (34–36). Endothelial responses to a high FGF23 level could induce suicidal red blood cell death (eryptosis) with a reactive rise in RDW (16,25). As has been shown in animal model, the presence of c-terminal FGF23 fragments may modulate FGF23 mediated effects and as such the role of endothelial cells on erythrocyte turnover can be influenced (37).

Additional mechanistic studies linking iron metabolism, FGF23 and red cell fate are relevant in CKD given the high risks of cardiovascular disease and death in these patients. Future research in which FGF23 levels are manipulated in order to influence RDW could possibly lead to a potential therapeutic intervention and improve the cardiovascular outcome in CKD patients. Conversely, it may be of importance to investigate the effect of influencing RDW (via iron manipulation) on FGF23 production and cleavage in osteocytes. If indeed cFGF23 is toxic to vessels, targets to interfere in the FGF23 secretion and catabolism could be helpful in preventing cardiovascular diseases.

Limitations of the study as a result of sample size need to be acknowledged. The size of our cohort which was based on the EPOCARES study is relatively small; yet the observed association between cFGF23 and RDW was robust in multivariate analyses. Furthermore, this study, performed among elderly people with CKD and CHF, may not be generalized to the entire CKD population. Finally, no cause-effect relationship can be established from this study due to its cross-sectional nature. The assumption that c-terminal FGF23 has its effect on the vascular wall is purely speculative, so we consider this a hypothesis-generating study that serves to fuel future prospective studies.

Strong points of our study include the fact that we measured both C-terminal and intact FGF23, which allowed us to obtain specific information on FGF23 cleaving, and the fact that we adjusted our analyses for markers of iron status and inflammation. However, this also yields a limitation, as the comparison of the results of the two assays measuring cFGF23 and iFGF23 is difficult due to the use of different units. Since the proportion of FGF23 that is cleaved is unknown, cFGF23 can only be reported as unit/volume. The two assays may detect different FGF23 epitopes and therefore in biological systems the affinity for these respective epitopes may differ and explain

limited linearity between these two ELISAs. This may apply in our EPOCARES subjects as well. However, our group has published results comparing the two assays in a range of concentrations and demonstrated a reasonable linearity of both assays (28). We decided to use iFGF23:cFGF23 ratio as a measure for the amount of cleaved FGF23 present, in accordance with recommendations by others (15).

In conclusion, our study demonstrates an association between cFGF23 and RDW but not iFGF23, suggesting that RDW is linked with FGF23 cleaving. Although iron deficiency and inflammation are known determinants of RDW as well as FGF23 metabolism, these factors only partly explained the association between RDW and FGF23. Further research is warranted to address additional mechanisms driving the association between FGF23 and red cell metabolism, and particularly RDW, in patients with CKD and CHF.

References

1. Scrutinio D, Passantino A, Santoro D, Catanzaro R (2011) The cardiorenal anaemia syndrome in systolic heart failure: prevalence, clinical correlates, and long-term survival. Eur J Heart Fail 13: 61-67. hfq167 [pii];10.1093/eurjhf/hfq167 [doi].

2. Smith GL, Lichtman JH, Bracken MB, Shlipak MG, Phillips CO, DiCapua P, Krumholz HM (2006) Renal impairment and outcomes in heart failure: systematic review and meta-analysis. J Am Coll Cardiol 47: 1987-1996. S0735-1097(06)00488-8 [pii];10.1016/j.jacc.2005.11.084 [doi].

3. Shlipak MG, Fried LF, Cushman M, Manolio TA, Peterson D, Stehman-Breen C, Bleyer A, Newman A, Siscovick D, Psaty B (2005) Cardiovascular mortality risk in chronic kidney disease: comparison of traditional and novel risk factors. JAMA 293: 1737-1745. 293/14/1737 [pii];10.1001/jama.293.14.1737 [doi].

4. Lippi G, Targher G, Montagnana M, Salvagno GL, Zoppini G, Guidi GC (2008) Relationship between red blood cell distribution width and kidney function tests in a large cohort of unselected outpatients. Scand J Clin Lab Invest 68: 745-748. 794905563 [pii];10.1080/00365510802213550 [doi].

5. Zoccali C, Yilmaz MI, Mallamaci F (2013) FGF23: A Mature Renal and Cardiovascular Risk Factor? Blood Purif 36: 52-57. 000351001 [pii];10.1159/000351001 [doi].

6. Farrow EG, Yu X, Summers LJ, Davis SI, Fleet JC, Allen MR, Robling AG, Stayrook KR, Jideonwo V, Magers MJ, Garringer HJ, Vidal R, Chan RJ, Goodwin CB, Hui SL, Peacock M, White KE (2011) Iron deficiency drives an autosomal dominant hypophosphatemic rickets (ADHR) phenotype in fibroblast growth factor-23 (Fgf23) knock-in mice. Proc Natl Acad Sci U S A 108: E1146-E1155. 1110905108 [pii];10.1073/pnas.1110905108 [doi].

7. Wolf M, Koch TA, Bregman DB (2013) Effects of iron deficiency anemia and its treatment on fibroblast growth factor 23 and phosphate homeostasis in women. J Bone Miner Res . 10.1002/jbmr.1923 [doi].

8. Bournier M, Tissot N, Mari S, Boucherez J, Lacombe E, Briat JF, Gaymard F (2013) Arabidopsis ferritin 1 (AtFer1) gene regulation by the phosphate starvation response 1 (AtPHR1) transcription factor reveals a direct molecular link between iron and phosphate homeostasis. J Biol Chem 288: 22670-22680. M113.482281 [pii];10.1074/jbc.M113.482281 [doi].

9. Fliser D, Kollerits B, Neyer U, Ankerst DP, Lhotta K, Lingenhel A, Ritz E, Kronenberg F, Kuen E, Konig P, Kraatz G, Mann JF, Muller GA, Kohler H, Riegler P (2007) Fibroblast growth factor 23 (FGF23) predicts progression of chronic kidney disease: the Mild to Moderate Kidney Disease (MMKD) Study. J Am Soc Nephrol 18: 2600-2608. ASN.2006080936 [pii];10.1681/ASN.2006080936 [doi].

10. Hsu HJ, Wu MS (2009) Fibroblast growth factor 23: a possible cause of left ventricular hypertrophy in hemodialysis patients. Am J Med Sci 337: 116-122. 10.1097/MAJ.0b013e3181815498 [doi];00000441-200902000-00009 [pii].

11. Gutierrez OM, Januzzi JL, Isakova T, Laliberte K, Smith K, Collerone G, Sarwar A, Hoffmann U, Coglianese E, Christenson R, Wang TJ, deFilippi C, Wolf M (2009) Fibroblast growth factor 23 and left ventricular hypertrophy in chronic kidney disease. Circulation 119: 2545-2552. CIRCULATIONAHA.108.844506 [pii];10.1161/CIRCULATIONAHA.108.844506 [doi].

12. Gutierrez OM, Mannstadt M, Isakova T, Rauh-Hain JA, Tamez H, Shah A, Smith K, Lee H, Thadhani R, Juppner H, Wolf M (2008) Fibroblast growth factor 23 and mortality among patients undergoing hemodialysis. N Engl J Med 359: 584-592. 359/6/584 [pii];10.1056/NEJMoa0706130 [doi].

13. Jean G, Terrat JC, Vanel T, Hurot JM, Lorriaux C, Mayor B, Chazot C (2009) High levels of serum fibroblast growth factor (FGF)-23 are associated with increased mortality in long haemodialysis patients. Nephrol Dial Transplant 24: 2792-2796. gfp191 [pii];10.1093/ndt/gfp191 [doi].

14. Baia LC, Humalda JK, Vervloet MG, Navis G, Bakker SJ, de Borst MH (2013) Fibroblast growth factor 23 and cardiovascular mortality after kidney transplantation. Clin J Am Soc Nephrol 8: 1968-1978. CJN.01880213 [pii];10.2215/CJN.01880213 [doi].

15. Wolf M, White KE (2014) Coupling fibroblast growth factor 23 production and cleavage: iron deficiency, rickets, and kidney disease. Curr Opin Nephrol Hypertens 23: 411-419. 10.1097/01.mnh.0000447020.74593.6f [doi].

16. Felker GM, Allen LA, Pocock SJ, Shaw LK, McMurray JJ, Pfeffer MA, Swedberg K, Wang D, Yusuf S, Michelson EL, Granger CB (2007) Red cell distribution width as a novel prognostic marker in heart failure: data from the CHARM Program and the Duke Databank. J Am Coll Cardiol 50: 40-47. S0735-1097(07)01270-3 [pii];10.1016/j.jacc.2007.02.067 [doi].

17. Forhecz Z, Gombos T, Borgulya G, Pozsonyi Z, Prohaszka Z, Janoskuti L (2009) Red cell distribution width in heart failure: prediction of clinical events and relationship with markers of ineffective erythropoiesis, inflammation, renal function, and nutritional state. Am Heart J 158: 659-666. S0002-8703(09)00551-1 [pii];10.1016/j.ahj.2009.07.024 [doi].

18. Pascual-Figal DA, Bonaque JC, Redondo B, Caro C, Manzano-Fernandez S, Sanchez-Mas J, Garrido IP, Valdes M (2009) Red blood cell distribution width predicts long-term outcome regardless of anaemia status in acute heart failure patients. Eur J Heart Fail 11: 840-846. hfp109 [pii];10.1093/eurjhf/hfp109 [doi].

19. Allen LA, Felker GM, Mehra MR, Chiong JR, Dunlap SH, Ghali JK, Lenihan DJ, Oren RM, Wagoner LE, Schwartz TA, Adams KF, Jr. (2010) Validation and potential mechanisms of red cell distribution width as a prognostic marker in heart failure. J Card Fail 16: 230-238. S1071-9164(09)01176-2 [pii];10.1016/j.cardfail.2009.11.003 [doi].

20. Tonelli M, Sacks F, Arnold M, Moye L, Davis B, Pfeffer M (2008) Relation Between Red Blood Cell Distribution Width and Cardiovascular Event Rate in People With Coronary Disease. Circulation 117: 163-168. CIRCULATIONAHA.107.727545 [pii];10.1161/CIRCULATIONAHA.107.727545 [doi].

21. Oh HJ, Park JT, Kim JK, Yoo DE, Kim SJ, Han SH, Kang SW, Choi KH, Yoo TH (2012) Red blood cell distribution width is an independent predictor of mortality in acute kidney injury patients treated with continuous renal replacement therapy. Nephrol Dial Transplant 27: 589-594. gfr307 [pii];10.1093/ndt/gfr307 [doi].

22. Patel KV, Ferrucci L, Ershler WB, Longo DL, Guralnik JM (2009) Red blood cell distribution width and the risk of death in middle-aged and older adults. Arch Intern Med 169: 515-523. 169/5/515 [pii];10.1001/archinternmed.2009.11 [doi].

23. Patel KV, Semba RD, Ferrucci L, Newman AB, Fried LP, Wallace RB, Bandinelli S, Phillips CS, Yu B, Connelly S, Shlipak MG, Chaves PH, Launer LJ, Ershler WB, Harris TB, Longo DL, Guralnik JM (2010) Red cell distribution width and mortality in older adults: a meta-analysis. J Gerontol A Biol Sci Med Sci 65: 258-265. glp163 [pii];10.1093/gerona/glp163 [doi].

24. Perlstein TS, Weuve J, Pfeffer MA, Beckman JA (2009) Red blood cell distribution width and mortality risk in a community-based prospective cohort. Arch Intern Med 169: 588-594. 169/6/588 [pii];10.1001/archinternmed.2009.55 [doi].

25. Emans ME, van der Putten K, van Rooijen KL, Kraaijenhagen RJ, Swinkels D, van Solinge WW, Cramer MJ, Doevendans PA, Braam B, Gaillard CA (2011) Determinants of red cell distribution width (RDW) in cardiorenal patients: RDW is not related to erythropoietin resistance. J Card Fail 17: 626-633. S1071-9164(11)00153-9 [pii];10.1016/j.cardfail.2011.04.009 [doi].

26. van der Putten K, Jie KE, Emans ME, Verhaar MC, Joles JA, Cramer MJ, Velthuis BK, Meiss L, Kraaijenhagen RJ, Doevendans PA, Braam B, Gaillard CA (2010) Erythropoietin treatment in patients with combined heart and renal failure: objectives and design of the EPOCARES study. J Nephrol 23: 363-368. 59A56384-BA5A-41AD-91C7-179279C019CD [pii].

27. Wish JB (2006) Assessing iron status: beyond serum ferritin and transferrin saturation. Clin J Am Soc Nephrol 1 Suppl 1: S4-S8. 1/Supplement_1/S4 [pii];10.2215/CJN.01490506 [doi].

28. Heijboer AC, Levitus M, Vervloet MG, Lips P, ter Wee PM, Dijstelbloem HM, Blankenstein MA (2009) Determination of fibroblast growth factor 23. Ann Clin Biochem 46: 338-340. acb.2009.009066 [pii];10.1258/acb.2009.009066 [doi].

29. Vervloet MG, van Zuilen AD, Heijboer AC, ter Wee PM, Bots ML, Blankestijn PJ, Wetzels JF (2012) Fibroblast growth factor 23 is associated with proteinuria and smoking in chronic kidney disease: an analysis of the MASTERPLAN cohort. BMC Nephrol 13: 20. 1471-2369-13-20 [pii];10.1186/1471-2369-13-20 [doi].

30. Wolf M (2012) Update on fibroblast growth factor 23 in chronic kidney disease. Kidney Int 82: 737-747. ki2012176 [pii];10.1038/ki.2012.176 [doi].

31. Mirza MA, Alsio J, Hammarstedt A, Erben RG, Michaelsson K, Tivesten A, Marsell R, Orwoll E, Karlsson MK, Ljunggren O, Mellstrom D, Lind L, Ohlsson C, Larsson TE (2011) Circulating fibroblast growth factor-23 is associated with fat mass and dyslipidemia in two independent cohorts of elderly individuals. Arterioscler Thromb Vasc Biol 31: 219-227. ATVBAHA.110.214619 [pii];10.1161/ATVBAHA.110.214619 [doi].

32. Munoz MJ, Isakova T, Ricardo AC, Xie H, Navaneethan SD, Anderson AH, Bazzano LA, Xie D, Kretzler M, Nessel L, Hamm LL, Negrea L, Leonard MB, Raj D, Wolf M (2012) Fibroblast growth factor 23 and Inflammation in CKD. Clin J Am Soc Nephrol 7: 1155-1162. CJN.13281211 [pii];10.2215/CJN.13281211 [doi].

33. Lippi G, Targher G, Montagnana M, Salvagno GL, Zoppini G, Guidi GC (2009) Relation between red blood cell distribution width and inflammatory biomarkers in a large cohort of unselected outpatients. Arch Pathol Lab Med 133: 628-632. 2008-0279-OAR1 [pii];10.1043/1543-2165-133.4.628 [doi].

34. Yilmaz MI, Sonmez A, Saglam M, Yaman H, Kilic S, Turker T, Unal HU, Gok M, Cetinkaya H, Eyileten T, Oguz Y, Caglar K, Vural A, Mallamaci F, Zoccali C (2013) Longitudinal analysis of vascular function and biomarkers of metabolic bone disorders before and after renal transplantation. Am J Nephrol 37: 126-134. 000346711 [pii];10.1159/000346711 [doi].

35. Yilmaz MI, Sonmez A, Saglam M, Yaman H, Kilic S, Demirkaya E, Eyileten T, Caglar K, Oguz Y, Vural A, Yenicesu M, Zoccali C (2010) FGF-23 and vascular dysfunction in patients with stage 3 and 4 chronic kidney disease. Kidney Int 78: 679-685. ki2010194 [pii];10.1038/ki.2010.194 [doi].

36. Mirza MA, Larsson A, Lind L, Larsson TE (2009) Circulating fibroblast growth factor-23 is associated with vascular dysfunction in the community. Atherosclerosis 205: 385-390. S0021-9150(09)00009-4 [pii];10.1016/j.atherosclerosis.2009.01.001 [doi].

37. Goetz R, Nakada Y, Hu MC, Kurosu H, Wang L, Nakatani T, Shi M, Eliseenkova AV, Razzaque MS, Moe OW, Kuro-o M, Mohammadi M (2010) Isolated C-terminal tail of FGF23 alleviates hypophosphatemia by inhibiting FGF23-FGFR-Klotho complex formation. Proc Natl Acad Sci U S A 107: 407-412. 0902006107 [pii];10.1073/pnas.0902006107 [doi].

Chapter 3

EFFECT OF FERRIC CARBOXYMALTOSE AND IRON DEXTRAN ON FGF23 METABOLISM AND SENSITIVITY IN HEALTHY AND UREMIC MICE

G.F. van Breda[1],M.H. de Borst[2], E.D. Keuning[3], W. Wickenhagen[4], T. Hoekstra[1], R. van der Swelm[5], H.W. van Essen[6], N. Bravenboer[6], M. Vervloet[1*] and C.A.J.M. Gaillard[7*]

1. Amsterdam UMC, Vrije Universiteit Amsterdam, Nephrology, Amsterdam Cardiovascular sciences, Amsterdam, Netherlands
2. University of Groningen, University Medical Center Groningen, Department of Internal Medicine, Division of Nephrology, Groningen, the Netherlands
3. Amsterdam UMC, Vrije Universiteit Amsterdam, Molecular Cell Biology and Immunology, Amsterdam, the Netherlands
4. Amsterdam UMC, Vrije Universiteit Amsterdam, Clinical Chemistry/endocrinology, Amsterdam, the Netherlands
5. Radboud University medical center, Department of Laboratory Medicine, Radboud institute for Molecular Life Sciences, Nijmegen, The Netherlands
6. Amsterdam UMC, Vrije Universiteit Amsterdam, Clinical Chemistry, Amsterdam, The Netherlands
7. UMC Utrecht, Division of Internal Medicine and Dermatology, University of Utrecht, Utrecht, the Netherlands

Manuscript in preparation

Abstract

Objective: High circulating levels of fibroblast growth factor (FGF23) in patients with chronic kidney disease (CKD) are associated with, and may induce cardiovascular disease. Iron deficiency, besides being a risk factor itself, increases FGF23 production. Iron repletion in turn can restore this overproduction, but remarkably some iron formulations induce temporary hypophosphatemia. The main goal of this study was to address the effect of modulation of iron status on FGF23 and downstream regulation of renal phosphate homeostasis. rFGF23 was administered to test the effect of different iron status on tubular sensitivity for FGF23. Studies were performed in healthy mice and mice with kidney failure.

Materials and methods: Mice were fed an iron sufficient (34,25 ppm) or deficient (0.01 ppm) diet for 6 weeks. Iron was administered as a single iv injection of 0.015 mg/g body weight ferric carboxymaltose (FCM) or iron dextran (ID). We determined the effects on plasma intact and C-terminal FG23, ferritin and phosphate, bone mRNA levels of FGF23 and klotho. rFGF23 was administered ip twice in a dose of 160 µg/kg during the stay in a metabolic cage and fractional phosphate excretion was determined. Experiments were performed in healthy mice and in 5/6 nephrectomized mice.

Results: Nephrectomized mice had significant kidney failure and iron administration resulted in significant increases in plasma ferritin levels. However, liver iron content did not differ between the two diet groups, suggesting only small differences in these groups with regard to iron status. Overall, dietary iron content nor iron supplementation changed FGF23 metabolism as reflected by its serum concentrations and bone expression pattern. In addition, rFGF23 did not result in a change in fractional phosphate excretion between experimental groups.

Conclusions: Substantial iron deficiency was not induced in this model, and hence no conclusion can be drawn in this regard to FGF23 metabolism or sensitivity**.** Iron loading in both normal and uremic mice by a single high dose of FCM or ID did not change iFGF23, cFGF23 and phosphate levels. In addition, iron loading did not change the sensitivity for rFGF23 based on fractional phosphate excretion.

Introduction

Fibroblast growth factor (FGF23) is an osteocyte-derived hormone that controls phosphate homeostasis by promoting renal phosphate excretion (1). FGF23 requires Klotho as a co-receptor to increase its affinity for the FGF receptor (FGFR) (2). Patients with advancing chronic kidney disease (CKD) have a progressive rise in FGF23 levels, which is considered to be the consequence of either phosphate exposure or FGF23 resistance due to loss of renal klotho. FGF23 is associated with CKD progression, cardiovascular events and mortality (3-5). Moreover, FGF23 has been suggested to be causally related to left ventricular hypertrophy (6) and therefore may qualify as a therapeutic target to prevent cardiovascular disease (CVD) in patients with CKD.

Recently iron deficiency was recognized as an environmental factor that stimulates FGF23 production in osteocytes (7-12). FGF23 is secreted as intact FGF23 (iFGF23) which is biologically active and can be cleaved by furin within osteocytes into biologically inactive c- and n-terminal FGF23 (cFGF23) (1). The exact role of iron on FGF23 production and cleavage is incompletely understood, especially in patients with CKD. Modulating iron status might impact cardiovascular risk due to the influence on FGF23 production and cleavage. Remarkably, different iron preparations can have diverging effects on serum iFGF23 concentrations and phosphate homeostasis, with some formulations like ferric carboxymaltose (FCM) occasionally induced transient hypophosphatemia, despite a generally declined FGF23 concentration after restoring iron depletion (13-17).

The goal of the present study is to obtain insight into the effects of varying iron status on FGF23 production, cleavage and sensitivity in mice with either normal kidney function or kidney failure. In addition, we studied the influence of intravenous iron on FGF23 cleavage. To reveal the mechanism behind the previously described different effects of several iron preparations on phosphate homeostasis, both FCM and iron dextran (ID) were given. Finally, cleavage-resistant recombinant FGF23 (rFGF23) was administered to examine differences of sensitivity for FGF23 at the tubular level for different iron formulations. Therefore, at the end of the experiments, a fixed dose of rFGF23 was administered and subsequently 24 hours urinary phosphate excretion and its fractional excretion was determined.

Materials and Methods

Ethical statement

This study was carried out after approval of the Central Authority for Scientific Procedures on Animals (CCD). All the procedures were conducted following the guidelines of the local Animal Ethical Committee, adhering the guidelines of the European animal welfare. All performed experiments were approved by the animal ethics board of the VU University Center (VUmc) and all efforts were made to reduce any potential suffering of the animals. Ethical approval was also based on pilot data from our lab that showed adequate induction of iron deficiency by iron-depleted diet, as reflected by significant decline of ferritin and increase in cFGF23 (data not shown).

Mice

Pathogen-free three-week old male and female C57Bl/6 mice were purchased from Charles River Laboratories and housed under standardized conditions in the animal facilities at the VUmc. After one-week acclimatization, mice were maintained on a standard diet containing 45 mg Fe/kg (Research Diets, AIN-76A, D09070191 with added iron, 34,25 ppm iron final concentration) or on an iron deficient diet (Research Diets, AIN-76A, D09070192, 0.1 ppm iron final concentration) for six weeks, previously described as inducing iron deficiency (18). All other nutritional factors were equal. To avoid potential contamination with iron, solid drinks were introduced and mice were housed in disposable plastic cages. Diets and water were introduced at weaning (age 4 weeks) and were provided at libitum throughout the study.

Design

This study was divided into two parts: the first part was performed in mice with normal kidney function, the second part in mice with kidney failure. To study the effects of different iron conditions on FGF23 metabolism and sensitivity in healthy mice, the following experimental groups were created, involving 108 mice with normal kidney function:

- Group 1: normal iron diet (n=9)
- Group 2: iron deficient diet (n=9)
- Group 3: iron sufficient diet followed by FCM (n=9)
- Group 4: iron deficient diet followed by FCM (n=9)
- Group 5: iron sufficient diet followed by iron dextran (n=9)
- Group 6: iron deficient diet followed by iron dextran (n=9)
- Group 7: normal iron diet ended by rFGF23 (n=9)
- Group 8: iron deficient diet ended by rFGF23 (n=9)

- Group 9: iron sufficient diet followed by FCM and ended by rFGF23 (n=9)
- Group 10: iron deficient diet followed by FCM and ended by rFGF23 (n=9)
- Group 11: iron sufficient diet followed by iron dextran and ended by rFGF23 (n=9)
- Group 12: iron deficient diet followed by iron dextran and ended by rFGF23 (n=9)

As it is currently unknown whether or how iron influences FGF23 metabolism in kidney failure, an acquired state of disturbed phosphate homeostasis and increased FGF23 concentrations, these experiments were repeated in mice with kidney failure induced by 5/6 nephrectomy, part two of the experiments. Based on a sample size calculation and taking into account the possible loss of animals due to surgical complications, there were 12 5/6 nephrectomy mice in every one of 12 groups, yielding a total number of 144 CKD mice in the second part of the study.

5/6 nephrectomy model

Partial nephrectomy (5/6Nx) was performed under standardized conditions as described previously (19, 20). Briefly, a small abdominal midline incision of the skin and muscles was made under general anesthesia (isoflurane) and preoperative analgesia (buprenorphine; Temgesic®; Schering-Plough, Houten, The Netherlands, 0.05 mg/kg intramuscular). The left kidney was decapsulated after which both the upper and lower pole were ablated by cauterization (High-temperature fine tip Cautery, Bovie Medical Corporation, Clearwater, FL, USA). Subsequently, in the same surgical session, the contralateral kidney was decapsulated and renal blood vessel and ureter were ligated, after which the entire kidney was removed. The abdomen was closed with sutures in two layers and all mice received subcutaneous injections of postoperative analgesia two days after surgery (Ketoprofen; Ketofen® (Merial SAS, Velserbroek, The Netherlands, 5 mg/kg). Sham-operated mice were used as controls and underwent the similar protocol including decapsulation of both kidneys, except for the renal ablation or extirpation.

Experimental procedures

Intra-venous tail vein injections included a single injection of ferric carboxymaltose (FCM, 0.015 mg/g body weight i.v. once) or iron dextran (ID, 0.015 mg/g body weight i.v. once) 1 day before sacrifice to induce iron repletion or iron overload. One day before sacrifice under total anesthesia (isoflurane), mice were placed into individual metabolic cages enabling 24 hrs urine collection. During these 24 hrs, mice received two intra-peritoneal injections of 160 µg/kg carrier-free cleavage-resistant recombinant mouse FGF23 (rFGF23; R&D Systems, Minneapolis, MN, USA; cat.no 2629-FG-CF) in a total volume of 100 µl to test the tubular sensitivity for FGF23. In rFGF23, modulation of the proteolytic cleavage site blocks the cleavage of FGF23, thereby preventing loss of FGF23 activity (21). Serum samples were obtained by tail bleeding or intracardiac exsanguination

at the end of the experiment. Blood was divided into EDTA-coagulated microtainers with added aprotinin to prevent degradation of FGF23 (22) and centrifuged 10 minutes at 1.800 G at 21 °C. Plasma samples were stored at -80 °C.

Biochemistry and urine samples

FGF23 levels were measured using both an iFGF23 ELISA that measures the intact active protein exclusively (Kainos Laboratories, Tokyo, Japan) and a murine cFGF23 ELISA that recognizes the full-length protein and its C-terminal cleavage fragments (Immutopics, Carlsbad, CA, USA). In order to estimate the amount of iFGF23 in relation to the total amount of FGF23 (i.e. intact FGF23 + c-terminal FGF23 as measured by the c-terminal assay), we calculated the iFGF23/cFGF23 ratio. For other assays, phosphate was measured using a molybdate UV assay on a Roche/Hitachi Cobas c 702 analyser (Roche-diagnostics, Basel, Swiss), creatinine was measured using an enzymatic method on a Roche/Hitachi Cobas c 702 analyser (Roche-diagnostics, Basel, Swiss), hemoglobin was measured colorimetric on a Sysmex XN9000 routine analyser (Sysmex corporation, Japan) and ferritin by mouse ELISA (Abcam, Cambrigde, MA, USA). Fractional phosphate excretion was calculated using the formula: [(urine phosphate in mmol/L) x (serum creatinine in mmol/L)]/[(serum phosphate in mmol/L) x (urine creatinine in mmol/L)].

Quantitative hepatic iron concentration

Harvested livers were frozen in liquid nitrogen and stored at -80°C. Small pieces of the livers (~100 mg) were weighed and homogenized. Liver tissue iron levels were determined using the chromogen bathophenanthroline as previously described (23). Iron concentrations were calculated by comparison to a standard curve of ferrous sulphate and corrected for protein concentration. Values were expressed as milligrams of iron per gram of dry weight.

RNA preparation and quantitative PCR of bone tissue

The proximal femurs were cleaned of soft tissue, pulverized in liquid nitrogen using the Freezer mill 6750 (Spex Certiprep, Metuchen, NY, USA) and extracted with Trizol (Invitrogen, Carlsbad, CA, USA). This was followed by a phenol extraction and a second trizol extraction according to the manufacturer's instructions. Possible DNA contamination was removed by DNAse incubation (Promega, Leiden, the Netherlands). The RNA pellet was dissolved in RNase-free water and stored at -80 °C until use. The yield of RNA was measured by Nanodrop spectrophotometer (NanoDrop® ND-1000 Spectrophotometer)(24).

One hundred ng of total RNA was reverse-transcribed using 10 ng/µl random primers (Roche, Basel, Switzerland) and 5 U/µl M-MLV Reverse Transcriptase (Promega) in a mixture containing

5 mM MgCl, 1x RT-buffer, 1 mM dNTPs each, 1M betaine and 0.40 U/µl RNAsin for 10 min at 25°C, 1h at 37°C and 5 min at 95°C in a total volume of 20 µl. For real-time qPCR reaction the cDNA was diluted 5 folds with RNase free water. For a 10 µL qPCR reaction, 2 µL of cDNA was mixed with a 5µL SYBR Green qPCR mastermix (Roche Diagnostics, Germany) and 2 µL H_2O. One microliter mixture of 0.5 µL reverse and 0.5µL forward primers (each 10 pmol/L) was added in the qPCR reaction. The PCR reaction consisted of an initial denaturation step of 1 cycle at 95 °C for 10 minutes, followed by an amplification step of 45 cycles as follows: 95 °C for 10 seconds, 60 °C for 5 seconds, 72 °C for 10 seconds. Primers were designed for the mouse genes: *FGF23*, *HIF1*α, *Furin*, *GalNT3* and a-*Klotho*. The Light Cycler 480 release 1.5.0 SP4 software (Roche, Germany) was used to analyze gene expression data. The gene expression was normalized with housekeeping genes *TBP* and *B2M* using the formula: $2^{-\Delta^{Cp}}$ (25).

Statistics

For statistical analysis, the SPSS software package version 20 was used (SPSS, IBM, Chicago, IL, USA). Descriptive data are presented as mean +/- SD or SEM. Differences between groups with normal distribution were analyzed using two-sided t-tests. P values < 0.05 were considered statistically significant.

Results

Induction of kidney failure and modulation of iron status

CKD mice had elevated levels of plasma creatinine compared to healthy mice (Table 1, P< 0.01). Plasma iFGF23 and cFGF23 levels increased significantly in CKD mice compared to non-CKD mice (both P< 0.01). Mice on an iron deficient diet had a statistically lower plasma ferritin level, while administration of intravenous iron (both FCM and ID) led to significantly higher ferritin levels. MCV levels were significant lower in mice fed an iron deficient diet, both in non-CKD and in CKD. RDW levels were higher in non-CKD mice with iron deficiency but not in CKD mice with iron deficiency. Liver iron content of mice fed an iron deficient, iron sufficient or iron loading were similar (figure 1). No mortality was observed after intravenous iron injection.

Table 1. Uremic condition and iron status of experimental mice

Group	Creat (umol/L)	iFGF23 (pg/ml)	cFGF23 (RU/ml)	Ferritin (ng/mL)	MCV (/um^3)	RWD (%)	Hb (g/dL)
Non-CKD	17.0 ± 7.7	228 ± 98	476 ± 202				
Low iron diet				570 ± 127*	50.8 ± 0.9*	26.1 ± 2.2*	9.7 ± 0.3
Normal iron diet				1195 ± 770	52.9 ± 1.2	21.3 ± 0.6	9.7 ± 0.2
Iron loading				1927 ± 696*	52.4 ± 0.9	21.4 ± 0.5	9.7 ± 0.2
CKD	26.0 ± 6.7	416 ± 208	1042 ± 479				
Low iron diet				550 ± 248*	48.4 ± 1.1*	21.9 ± 2.6	8.2 ± 1.5
Normal iron diet				936 ± 439	49.4 ± 0.8	22.1 ± 0.9	9.0 ± 0.4
Iron loading				1450 ± 421*	49.4 ± 0.8	21.5 ± 1.0	8.6 ± 0.6*

Data expressed as mean ±SD, *P< 0.05 vs normal iron diet. Creat= creatinine, iFGF23= intact FGF23, cFGF23= c-terminal FGF23, MCV= mean corpuscular volume, RDW= red cell distribution width, Hb= hemoglobin.

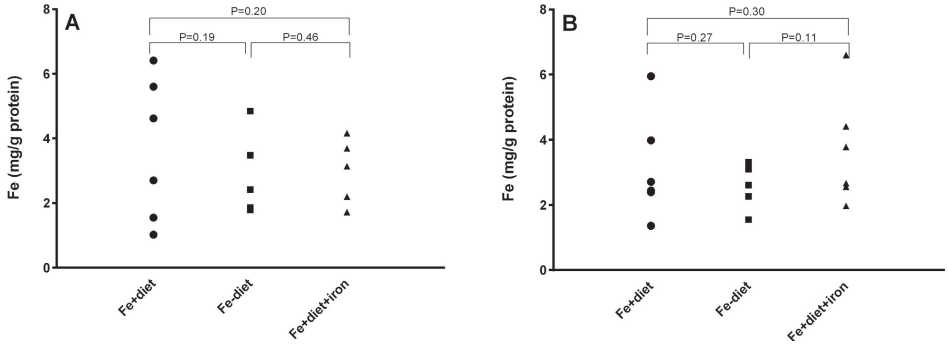


Figure 1. Liver iron contents in (•) normal iron-diet mice, (▪) mice with iron-deficient diet and (▲) iron loaded mice in non-CKD (A) and CKD (B).

Effects of iron modulation on FGF23 metabolism in healthy mice

To assess the effects of dietary iron content on FGF23 metabolism in healthy mice, both iFGF23 and cFGF23 levels, iFGF23/cFGF23 ratio and phosphate levels were compared between mice fed a standard-iron diet vs an iron-deficient diet (table 2, upper part). Body weight and kidney function were similar between groups (data not shown). All above mentioned parameters were not different between the two groups.

To assess the effect of different iron supplements on FGF23 metabolism in healthy mice, intravenous FCM or ID was given in both healthy mice with standard-iron diet and mice with iron deficient diet. Overall, no effects on iFGF23 and cFGF23 were found following intravenous iron administration of either formulation, except a statistically significant increase in iFGF23 levels in the group with normal iron status and ID supplementation, i.e. in iron loaded mice. There was a statistically significant lower plasma phosphate level in mice with low iron diet that received ID. The iFGF23/cFGF23 ratio did not show significant differences between the different groups (table 2, upper part).

Effects of iron modification on FGF23 metabolism in CKD mice

We subsequently assessed the effects of dietary iron status on FGF23 metabolism in 5/6 nephrectomy mice (table 2, lower part).

Body weight and kidney function were similar between the groups (data not shown). Phosphate levels remained stable. cFGF23 and iFGF23 levels did not differ between low-iron diet and standard-iron diet in CKD.

To assess the effects of FCM and ID on FGF23 in a uremic environment, these iron supplements were given in 5/6 nephrectomy mice. In mice with CKD, no effects on iFGF23 and cFGF23 were shown following intravenous iron administration. In addition, the phosphate levels were similar in the experimental groups. The iFGF23/cFGF23 ratio also did not show statistically significant differences between the different groups.

Table 2. FGF23 and phosphate metabolism for different iron status and kidney function

	iFGF23 (pg/ml)	cFGF23 (RU/ml)	iFGF23/cFGF23	Phosphate (mmol/L)
Non CKD				
Normal iron diet	142 (25)	356 (151)	0.43 (0.11)	2.49 (1.14)
Low iron diet	164 (34)	464 (276)	0.40 (0.14)	2.76 (0.92)
Normal iron diet+ FCM	144 (33)	381 (189)	0.42 (0.09)	2.53 (0.78)
Normal iron diet+ ID	189 (52)#	458 (177)	0.43 (0.08)	2.55 (0.40)
Low iron diet + FCM	181 (37)	435 (130)	0.43 (0.07)	2.70 (0.68)
Low iron diet + ID	193 (65)	588 (295)	0.36 (0.10)	2.00 (0.47)#
CKD				
Normal iron diet	442 (225)	1242 (670)	0.37 (0.12)	2.09 (0.59)
Low iron diet	446 (194)	1147 (458)	0.40 (0.14)	1.99 (0.63)
Normal iron diet + FCM	433 (210)	1126 (506)	0.37 (0.07)	1.98 (0.37)
Normal iron diet + ID	331 (94)	836 (303)	0.42 (0.11)	2.09 (0.29)
Low iron diet + FCM	395 (137)	975 (340)	0.42 (0.11)	1.64 (0.26)
Low iron diet + ID	357 (111)	1028 (583)	0.41 (0.15)	1.58 (0.34)

Data expressed as mean ±SD, #P< 0.05 loading vs normal. FCM= ferric carboxymaltose, ID= iron dextran.

The effect of different iron conditions on the sensitivity for recombinant FGF23

To test whether different iron conditions are of influence on the sensitivity for FGF23, intraperitoneal rFGF23 was given during the stay in metabolic cages just before sacrifice. Overall, fractional phosphate excretion was not affected by rFGF23 in experimental groups. In non-CKD mice, a significant decrease in phosphate excretion was seen in mice fed a normal iron diet, supplemented by FCM and after rFGF23 injection compared to mice with non-CKD mice with normal iron diet. In CKD mice, the group with iron deficient diet and ID supplementation had significant higher phosphate excretion compared to mice with iron deficient diet without supplementation of iron and rFGF23. Since the amount of urine produced during the stay in

metabolic cages varied, the absolute amount of phosphate excretion was calculated (figure 3). Overall, the absolute phosphate excretion did not change by administering rFGF23, except for healthy mice with iron deficient diet with or without ID supplementation and rFGF23.

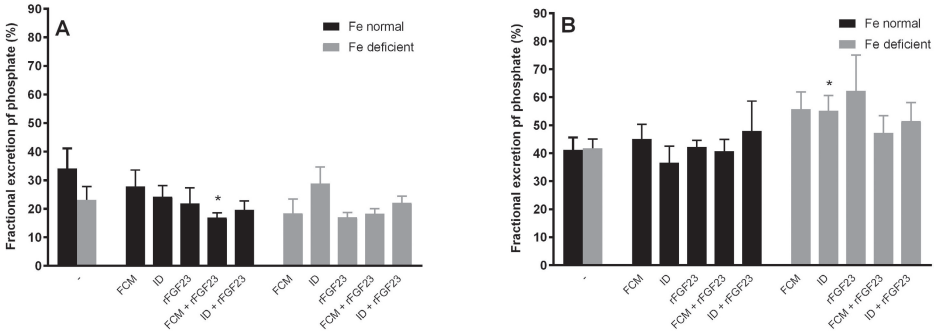


Figure 2. Fractional phosphate excretion in (A) non-CKD mice and (B) CKD mice. Black bars: mice fed a normal iron diet, grey bars: mice fed an iron deficient diet. FCM= ferric carboxymaltose, ID= iron dextran, rFGF23= recombinant FGF23. * P< 0.05 vs mice with same diet and without supplementation of iron or rFGF23.

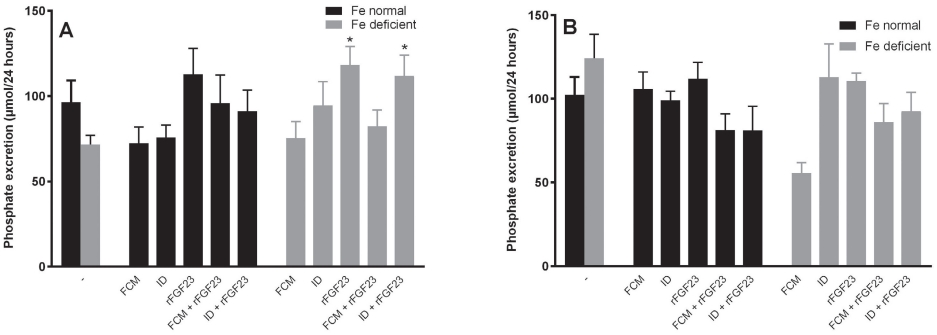


Figure 3. Absolute phosphate excretion per 24 hours in (A) non-CKD mice and (B) CKD mice. * P< 0.05 vs mice with same diet and without supplementation of iron or rFGF23.

Bone mRNA expression in different iron conditions

In addition to determining FGF23 in blood, FGF23 expression on bone tissue level was examined. Figure 3 shows the mRNA expression of FGF23, klotho, HIF1α and furin in bone. No differences in mRNA expression was found for any of these genes.

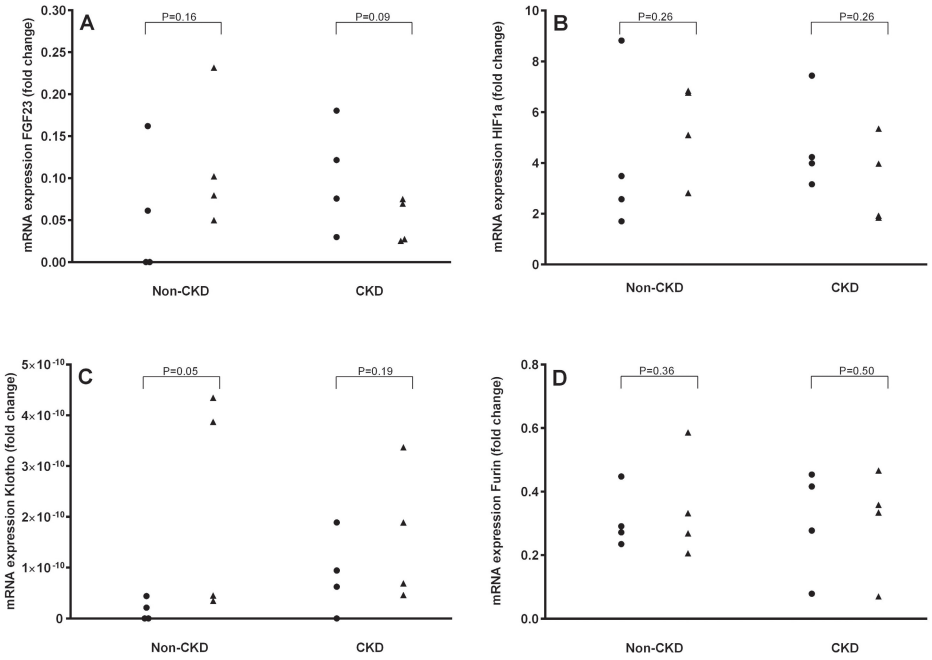


Figure 4: Pooled data (three bones per dot) of mRNA expression of (A) FGF23, (B) klotho, (C) HIF1α and (D) furin in proximal femurs of (•) iron deficient mice and (▲) iron replete mice.

Discussion

In this study, iron deficient diet, which induced hematological evidence of functional iron deficiency, did not induce changes in FGF23 metabolism or tubular handling of phosphate in mice. In addition, iron loading by either FCM or ID, did not modify these parameters of FGF23 and phosphate homeostasis. The absence of an effect of iron depletion on FGF23 metabolism was similar in healthy and CKD mice. Moreover, these differences in iron status did not induce differences in expression of FGF23, klotho, HIF1α and furin in bone tissue. Finally, kidney sensitivity for FGF23, as assessed by administration of cleavage-resistant rFGF23, was unaffected by iron status or CKD. Although plasma parameters (ferritin, MCV and RDW) support the validity of our experimental model, in this study iron deficiency does not induce changes in FGF23 metabolism.

Iron administration and FGF23

The results of this study are not in keeping with previously published reports (11, 18, 26). Mature mice with normal kidney function showed increased cFGF23 levels, normal iFGF23 levels and increased bone FGF23 mRNA expression in the setting of iron deficiency. However, although the effect of iron deficiency on FGF23 metabolism has been examined in the setting of normal

kidney function, the consequences of different iron status during impaired kidney function are less well described. Hanudel et at. placed wild type mice on 8-week diets with low or standard iron concentrations, with or without adenine to induce CKD (27). They showed that in both control and CKD mice, a low iron diet caused increased bone FGF23 mRNA expression and cFGF23 levels. Based on the concentrations of cFGF23 and iFGF23, they concluded that during CKD, both FGF23 production and cleavage were increased. This contradicts our study results, which showed no effect of iron on FGF23 metabolism. As expected by the results of the serum FGF23 levels, iron deficiency was not associated with increased mRNA FGF23 levels. It is well established that iron deficiency is associated with stabilization of hypoxia-inducible factor α (HIF 1α), which can increase transcription of FGF23 (11, 26). HIF1α also upregulates furin, which cleaves FGF23 (26, 28, 29). Based on these facts, HIF1α could contribute to iron deficiency-mediated coupling of increased FGF23 production and cleavage. In our study however, no upregulation of HIF 1α occurred. These negative results of iron status on mRNA of both HIF 1α and FGF23 suggest that the induction of a substantial iron deficiency failed in our study.

Limited by the amount of blood that could be extracted from mice, a choice in serum chemistries had been made in advance. Ferritin was chosen as the most reliable serum parameter for iron status. After 6 weeks of iron deficient diet, mice indeed had significantly lower levels of ferritin. The difference in ferritin levels between the two groups of dietary intervention in our study were even more pronounced than in other studies with an experimental mice model with iron deficiency (26). Additionally, MCV decreased and RDW increased in all groups, providing additional evidence for the existence of relevant iron deficiency. Motivated by the unexpected results, additional evidence for iron depletion was collected by measuring iron content in the liver. In contrast to ferritin levels in serum, iron content in the liver did not differ between groups. Previous research has shown that measurement of hepatic iron concentrations is accurate for detecting systemic iron deficiency or sufficiency (30, 31). This somehow questions if iron deficiency in our model was severe enough, or of sufficient duration, to have an impact on FGF23 metabolism or sensitivity. However, iron depleted diets were able to impact on ferritin levels, MCV and RDW, all reflecting biological significance, as shown in table 1. The experimental diets were obtained from Research Diets, Inc. This choice was made based on excellent results in other research in terms of adequately reaching contrasts in iron status (11, 18). In these reports, iron deficiency was not only demonstrated by serum ferritin levels and FGF23 mRNA expression but also by serum iron chemistry, hepcidin mRNA or renal EPO and transferrin receptor type1 mRNA expression, but not by measuring iron content as we did. Farrow et al. placed the mice on the same iron deficient and sufficient diet for 8 weeks, which is 2 weeks longer than our experimental groups (11) and showed statistically significant differences in both iron and iFGF23 and cFGF23 parameters. However, in

the study of Clinkenbeard et al (18), iron depletion was carried out by providing low-iron diet for 4 weeks only, which also induced significant differences in outcome parameters. These results and our pilot study, which showed statistically significant differences in ferritin, iFGF23 and cFGF23 levels after 6 weeks of low- and normal-iron diet, justified the set-up of our study exposing animals to 6 instead of 4 weeks of iron-depleted diets. In a subsequent study we may have to decide to extend this duration in order to gain more contrast in iron levels in our experimental model.

Iron administration and hypophosphatemia

Hypophosphatemia occurring after iv iron administration has been observed with several iron formulations, such as FCM, saccharated iron oxide and iron polymaltose (12, 15, 32-35), but not with ID or ferumoxytol (33, 36). Due to the different effects of these iron preparations, we chose FCM and ID for iron supplementation in our study. However, in contrast to previous results, in our study the only statistically significant decrease of serum phosphate level was in the non-CKD group on iron-deplete diet receiving ID. Given the relatively high number of groups in our study, this latter decline of phosphate in a specific group is probably there because of chance, as a consequence of multiple testing. The fact that phosphate does not decrease after iron administration does correspond to the absence of a change in iFGF23

Effect of iron on sensitivity for FGF23

As described, the effect of iron on FGF23 production and cleavage has been subject of many research projects. However, it is unknown whether iron affects the sensitivity for biologically active FGF23, which could be another explanation of changes in phosphate homeostasis. To get insight into this potential mechanism, rFGF23 (21) was given 24 hours before sacrifice. To measure tubular phosphate handling, mice were placed in metabolic cages to collect 24-hour urine samples. Even though the results from the analysis to the effect of rFGF23 on fractional phosphate excretion suggest some significant differences (figure 2 and 3), these differences have no relevance and could be explained by multiple testing. Therefore, no clear pattern can be detected and unfortunately our study does not contribute to a conclusion about the effect of different iron conditions on FGF23 sensitivity. Based on previous research with rFGF23 in our group (37) we chose carrier free rFGF23 (rFGF-CF) to assess the effects of systemic administration of cleavage resistant FGF23. The rFGF23-CF does not contain Bovine Serum Albumin (BSA) as a carrier protein with enhances protein stability, increases shelf-life, and allows the recombinant protein to be stored at a more dilute concentration. However, previous studies in which rFGF23 was administered, used addition of BSA (6). In our study, we dissolved rFGF23-CF in PBS and stored it into the refrigerator. During the stay in metabolic cages, this solution of rFGF23-CF and

PBS was used. Based on our results, it appears as if activity of rFGF23 was lost, which could explain the absence of biological effects of rFGF23 on phosphate excretion.

Implications for treatment and future research

This study contradicts previous studies, and questions the existence of a straightforward relation between iron deficiency and FGF23 production. However, our study may be somewhat flawed, because some data indicate that we induced modest iron deficiency only. Therefore, additional mechanistic studies are needed to unravel the mechanisms of iron depletion on FGF23 production (if it exists) and cleavage in osteocytes. Although there were no clear differences in iFGF23 and cFGF23 in the experimental groups, these data could have mechanistic importance. Our data and previous research may be reconciled with the assumption of the existence of a lower threshold below which iron deficiency does affect FGF23 transcription and cleavage. If such a threshold does exist, it could assist in clinical decision making with regard to the timing and dosing of iron supplementation in patients with CKD. The mechanism explaining the diverging effect of different iron preparations remains unresolved and should be clarified in order to prescribe a formulation that fits the patient. If there is more insight into the effects of different iron preparations, clinical trials should determine if and which oral or intravenous iron supplementation will not only improve iron parameters and anemia, but also influence FGF23 levels. The ultimate goal will be to study the influence of iron on hard clinical outcomes, mediated by FGF23 or not.

Strengths and limitations of this study

The strength of this study includes the fact that an extensive study design has been set up with 2 different iron formulations given in both healthy and CKD mice. FGF23 stabilization was accomplished by adding aprotinin to the tubes, a serine protease inhibitor, immediately after collection. Plasma samples may be less unsuitable for measurement of intact FGF23 unless stabilized with a protease inhibitor (22). In addition, blood was centrifuged within a time frame of 10 minutes after withdrawal to further limit the risk of FGF23 degradation. Our study does not contribute to the clarification of the mechanism behind the effect of iron on FGF23 production and cleavage. The most likely explanation is the fact that the degree of iron deficiency was too limited which is underlined by the absence of differences in liver iron content. However, measuring liver iron content has not been performed in other studies examining the effects of iron on FGF23 and therefore the degree of iron deficiency on tissue level is unknown in those studies. Associations between serum ferritin and tissue iron have been reported in hemodialysis patients who received intravenous iron . However, results were conflicting: Ali et al (38) found a positive correlation of serum ferritin concentrations with semi quantitative scores for hepatosplenic iron deposits in 36 patients. Fleming et al (39) showed a positive correlation between hepatic

iron content and cumulative iron dose, but no significant correlation between hepatic iron and plasma ferritin in 22 patient receiving dialysis. Before starting subsequent research, we need to re-validate our experimental model to make sure the prescribed diet has clear effects on iron storage and availability in mice. In addition, we should try to measure both ferritin and iron to confirm iron deficiency.

The dose of rFGF23 (160 ug/kg) was chosen based on a pilot experiment in which this dose demonstrated a serum phosphate lowering effect after administration of rFGF23 twice with a 12-hour interval, using peritoneal injections (40). However, in contrast to Faul et al (6), we didn't confirm in vivo biological activity in our experimental model. Although the dose of rFGF23 given in our study is considerably higher than in the study of Faul et al (160 ug/kg vs 40 ug/kg), it is noticeable that in our study rFGF23 was given only twice. Studies in which biological effect had been seen, rFGF23 has been given for several days (6, 41, 42). However, some if these studies were focused on structural tissue changes, for which it is conceivable that more prolonged exposure is a prerequisite, while we studied the functional properties of FGF23. Nevertheless, it remains possible that 2 injections of rFGF23 in a relatively short period before sacrifice are probably not sufficient to cause an optimal biological effect and in a future validation study, the optimal duration of rFGF23 injections should be clarified.

Conclusions

In sum, different iron conditions had no effect on iFGF23 and cFGF23 levels in healthy and CKD mice. A single intravenous iron injection of either FCM or ID did not alter the amount and cleavage of FGF23. In addition, the sensitivity for rFGF23 was not changed by different iron status. Although the model of iron deficiency seems to be correct based on significant differences in ferritin levels, there is doubt whether enough iron depletion has been reached. Subsequent research has to be done after we have made sure that sufficient iron deficiency has been achieved in our next experimental model.

References

1. Wolf M. Update on fibroblast growth factor 23 in chronic kidney disease. Kidney Int. 2012;82(7):737-47.

2. Urakawa I, Yamazaki Y, Shimada T, Iijima K, Hasegawa H, Okawa K, et al. Klotho converts canonical FGF receptor into a specific receptor for FGF23. Nature. 2006;444(7120):770-4.

3. Gutierrez OM, Mannstadt M, Isakova T, Rauh-Hain JA, Tamez H, Shah A, et al. Fibroblast growth factor 23 and mortality among patients undergoing hemodialysis. N Engl J Med. 2008;359(6):584-92.

4. Isakova T, Xie H, Yang W, Xie D, Anderson AH, Scialla J, et al. Fibroblast growth factor 23 and risks of mortality and end-stage renal disease in patients with chronic kidney disease. JAMA. 2011;305(23):2432-9.

5. Fliser D, Kollerits B, Neyer U, Ankerst DP, Lhotta K, Lingenhel A, et al. Fibroblast growth factor 23 (FGF23) predicts progression of chronic kidney disease: the Mild to Moderate Kidney Disease (MMKD) Study. J Am Soc Nephrol. 2007;18(9):2600-8.

6. Faul C, Amaral AP, Oskouei B, Hu MC, Sloan A, Isakova T, et al. FGF23 induces left ventricular hypertrophy. J Clin Invest. 2011;121(11):4393-408.

7. Econs MJ, McEnery PT. Autosomal dominant hypophosphatemic rickets/osteomalacia: clinical characterization of a novel renal phosphate-wasting disorder. J Clin Endocrinol Metab. 1997;82(2):674-81.

8. Imel EA, Hui SL, Econs MJ. FGF23 concentrations vary with disease status in autosomal dominant hypophosphatemic rickets. J Bone Miner Res. 2007;22(4):520-6.

9. Imel EA, Peacock M, Gray AK, Padgett LR, Hui SL, Econs MJ. Iron modifies plasma FGF23 differently in autosomal dominant hypophosphatemic rickets and healthy humans. J Clin Endocrinol Metab. 2011;96(11):3541-9.

10. Braithwaite V, Jarjou LM, Goldberg GR, Prentice A. Iron status and fibroblast growth factor-23 in Gambian children. Bone. 2012;50(6):1351-6.

11. Farrow EG, Yu X, Summers LJ, Davis SI, Fleet JC, Allen MR, et al. Iron deficiency drives an autosomal dominant hypophosphatemic rickets (ADHR) phenotype in fibroblast growth factor-23 (Fgf23) knock-in mice. Proc Natl Acad Sci U S A. 2011;108(46):E1146-E55.

12. Wolf M, Koch TA, Bregman DB. Effects of iron deficiency anemia and its treatment on fibroblast growth factor 23 and phosphate homeostasis in women. J Bone Miner Res. 2013.

13. Shimizu Y, Tada Y, Yamauchi M, Okamoto T, Suzuki H, Ito N, et al. Hypophosphatemia induced by intravenous administration of saccharated ferric oxide: another form of FGF23-related hypophosphatemia. Bone. 2009;45(4):814-6.

14. Prats M, Font R, Garcia C, Cabre C, Jariod M, Vea AM. Effect of ferric carboxymaltose on serum phosphate and C-terminal FGF23 levels in non-dialysis chronic kidney disease patients: post-hoc analysis of a prospective study. BMC Nephrol. 2013;14:167.

15. Schouten BJ, Hunt PJ, Livesey JH, Frampton CM, Soule SG. FGF23 elevation and hypophosphatemia after intravenous iron polymaltose: a prospective study. J Clin Endocrinol Metab. 2009;94(7):2332-7.

16. Auerbach M, Ballard H. Clinical use of intravenous iron: administration, efficacy, and safety. Hematology Am Soc Hematol Educ Program. 2010;2010:338-47.

17. Wikstrom B, Bhandari S, Barany P, Kalra PA, Ladefoged S, Wilske J, et al. Iron isomaltoside 1000: a new intravenous iron for treating iron deficiency in chronic kidney disease. J Nephrol. 2011;24(5):589-96.

18. Clinkenbeard EL, Farrow EG, Summers LJ, Cass TA, Roberts JL, Bayt CA, et al. Neonatal iron deficiency causes abnormal phosphate metabolism by elevating FGF23 in normal and ADHR mice. J Bone Miner Res. 2014;29(2):361-9.

19. Bro S, Bentzon JF, Falk E, Andersen CB, Olgaard K, Nielsen LB. Chronic renal failure accelerates atherogenesis in apolipoprotein E-deficient mice. J Am Soc Nephrol. 2003;14(10):2466-74.

20. Stitt-Cavanagh EM, Faour WH, Takami K, Carter A, Vanderhyden B, Guan Y, et al. A maladaptive role for EP4 receptors in podocytes. J Am Soc Nephrol. 2010;21(10):1678-90.

21. Frishberg Y, Ito N, Rinat C, Yamazaki Y, Feinstein S, Urakawa I, et al. Hyperostosis-hyperphosphatemia syndrome: a congenital disorder of O-glycosylation associated with augmented processing of fibroblast growth factor 23. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2007;22(2):235-42.

22. Smith ER, Ford ML, Tomlinson LA, Weaving G, Rocks BF, Rajkumar C, et al. Instability of fibroblast growth factor-23 (FGF-23): implications for clinical studies. Clin Chim Acta. 2011;412(11-12):1008-11.

23. Torrance JD, Bothwell TH. A simple technique for measuring storage iron concentrations in formalinised liver samples. S Afr J Med Sci. 1968;33(1):9-11.

24. Reijnders CM, van Essen HW, van Rens BT, van Beek JH, Ylstra B, Blankenstein MA, et al. Increased expression of matrix extracellular phosphoglycoprotein (MEPE) in cortical bone of the rat tibia after mechanical loading: identification by oligonucleotide microarray. PloS one. 2013;8(11):e79672.

25. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome biology. 2002;3(7):Research0034.

26. David V, Martin A, Isakova T, Spaulding C, Qi L, Ramirez V, et al. Inflammation and functional iron deficiency regulate fibroblast growth factor 23 production. Kidney Int. 2016;89(1):135-46.

27. Hanudel MR, Chua K, Rappaport M, Gabayan V, Valore E, Goltzman D, et al. Effects of dietary iron intake and chronic kidney disease on fibroblast growth factor 23 metabolism in wild-type and hepcidin knockout mice. American journal of physiology Renal physiology. 2016;311(6):F1369-f77.

28. McMahon S, Grondin F, McDonald PP, Richard DE, Dubois CM. Hypoxia-enhanced expression of the proprotein convertase furin is mediated by hypoxia-inducible factor-1: impact on the bioactivation of proproteins. The Journal of biological chemistry. 2005;280(8):6561-9.

29. Silvestri L, Pagani A, Camaschella C. Furin-mediated release of soluble hemojuvelin: a new link between hypoxia and iron homeostasis. Blood. 2008;111(2):924-31.

30. Trinder D, Olynyk JK, Sly WS, Morgan EH. Iron uptake from plasma transferrin by the duodenum is impaired in the Hfe knockout mouse. Proceedings of the National Academy of Sciences of the United States of America. 2002;99(8):5622-6.

31. Fleming RE, Migas MC, Holden CC, Waheed A, Britton RS, Tomatsu S, et al. Transferrin receptor 2: continued expression in mouse liver in the face of iron overload and in hereditary hemochromatosis. Proceedings of the National Academy of Sciences of the United States of America. 2000;97(5):2214-9.

32. Van Wyck DB, Mangione A, Morrison J, Hadley PE, Jehle JA, Goodnough LT. Large-dose intravenous ferric carboxymaltose injection for iron deficiency anemia in heavy uterine bleeding: a randomized, controlled trial. Transfusion. 2009;49(12):2719-28.

33. Hussain I, Bhoyroo J, Butcher A, Koch TA, He A, Bregman DB. Direct Comparison of the Safety and Efficacy of Ferric Carboxymaltose versus Iron Dextran in Patients with Iron Deficiency Anemia. Anemia. 2013;2013:169107.

34. Mani LY, Nseir G, Venetz JP, Pascual M. Severe hypophosphatemia after intravenous administration of iron carboxymaltose in a stable renal transplant recipient. Transplantation. 2010;90(7):804-5.

35. Okada M, Imamura K, Iida M, Fuchigami T, Omae T. Hypophosphatemia induced by intravenous administration of Saccharated iron oxide. Klinische Wochenschrift. 1983;61(2):99-102.

36. Vadhan-Raj S, Strauss W, Ford D, Bernard K, Boccia R, Li J, et al. Efficacy and safety of IV ferumoxytol for adults with iron deficiency anemia previously unresponsive to or unable to tolerate oral iron. American journal of hematology. 2014;89(1):7-12.

37. Pulskens WP, Verkaik M, Sheedfar F, van Loon EP, van de Sluis B, Vervloet MG, et al. Deregulated Renal Calcium and Phosphate Transport during Experimental Kidney Failure. PloS one. 2015;10(11):e0142510.

38. Ali M, Rigolosi R, Fayemi AO, Braun EV, Frascino J, Singer R. Failure of serum ferritin levels to predict bone-marrow iron content after intravenous iron-dextran therapy. Lancet. 1982;1(8273):652-5.

39. Fleming LW, Hopwood D, Shepherd AN, Stewart WK. Hepatic iron in dialysed patients given intravenous iron dextran. Journal of clinical pathology. 1990;43(2):119-24.

40. de Jong MA, Mirkovic K, Mencke R, Hoenderop JG, Bindels RJ, Vervloet MG, et al. Fibroblast growth factor 23 modifies the pharmacological effects of angiotensin receptor blockade in experimental renal fibrosis. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2017;32(1):73-80.

41. Verkaik M, Oranje M, Abdurrachim D, Goebel M, Gam Z, Prompers JJ, et al. High Fibroblast Growth Factor 23 concentrations in experimental renal failure impair calcium handling in cardiomyocytes. Physiological reports. 2018;6(7):e13591.

42. Verkaik M, Juni RP, van Loon EPM, van Poelgeest E, Kwekkeboom RFJ, Gam Z, et al. FGF23 impairs peripheral microvascular function in renal failure. American journal of physiology Heart and circulatory physiology. 2018.

Chapter 4

CARDIAC HEPCIDIN EXPRESSION ASSOCIATES WITH INJURY INDEPENDENT OF IRON

G.F. van Breda[1], L.G. Bongartz[2], W. Zhuang[3], R.P.L. van Swelm[4], J. Pertijs[5], B. Braam[3], M.J. Cramer[2], D.W. Swinkels[4], P.A. Doevendans[2], M.C. Verhaar[6], R. Masereeuw[7,] J.A. Joles[6], C.A. Gaillard[8]

1. Department of Nephrology and ICaR-VU, VUMC, Amsterdam, the Netherlands
2. Department of Cardiology, UMCU, Utrecht, the Netherlands
3. Department of Nephrology and Immunology, University of Alberta, Edmonton, Alberta, Canada
4. Department of Laboratory Medicine, Radboud UMC, Nijmegen, the Netherlands
5. Department of Pharmacology and Toxicology, Radboud UMC, Nijmegen, the Netherlands
6. Department of Nephrology and Hypertension, UMCU, Utrecht, the Netherlands
7. Department of pharmaceutical sciences, UMCU, Utrecht, the Netherlands
8. Department of Nephrology, University Medical Center Groningen, Groningen, the Netherlands

Am J Nephrol. 2016 Nov; 44(5): 368-378

Abstract

Background: Hepcidin regulates systemic iron homeostasis by downregulating the iron exporter ferroportin. Circulating hepcidin is mainly derived from the liver but hepcidin is also produced in the heart. We studied differential and local regulation of hepcidin gene expression in response to myocardial infarction (MI) and/or chronic kidney disease (CKD). We hypothesized that cardiac hepcidin gene expression is induced by and regulated to severity of cardiac injury, either through direct (MI) or remote (CKD) stimuli, and through increased local iron content.

Methods: Nine weeks after subtotally nephrectomy (SNX) or sham surgery (CON), rats were subjected to coronary ligation (CL) or sham surgery to realize four groups: CON, SNX, CL and SNX+CL. In week 16, gene expression of hepcidin, iron and damage markers in cardiac and liver tissue was assessed by qPCR and ferritin protein expression was studied by immunohistochemistry.

Results: Cardiac hepcidin mRNA expression was increased two-fold in CL ($P= 0.03$) and threefold in SNX ($P= 0.01$). Cardiac ferritin staining was not different among groups. Cardiac hepcidin mRNA expression correlated with mRNA expression levels of brain natriuretic peptide ($\beta= 0.734$, $P< 0.001$) and connective tissue growth factor ($\beta= 0.431$, $P= 0.02$). In contrast, liver hepcidin expression was unaffected by SNX and CL alone, while it was 50% decreased in SNX+CL ($P< 0.05$). Hepatic ferritin immunostaining was not different among groups.

Conclusions: Our data indicate differences in hepcidin regulation in liver and heart and suggest a role for injury rather than iron as driving force for cardiac hepcidin expression in renocardiac failure.

Introduction

Iron-dependent modulation of energy metabolism is important in tissues with high metabolic demand, such as the myocardium (1). The mechanisms involved in local iron metabolism are of critical importance since in the heart both iron overload and iron deficiency has poor outcomes [2-4]. Hepcidin is the main regulatory protein of systemic iron metabolism. It is mainly produced in the liver and binds to ferroportin (Fpn-1), a cellular iron exporter, resulting in internalization and degradation of the complex (5). Hepcidin thus inhibits cellular iron efflux from enterocytes, macrophages and hepatocytes (6). Because hepcidin synthesis is primarily controlled at the transcriptional level (7), hepcidin antimicrobial peptide (Hamp) mRNA expression level is a good indication of the amount of hepcidin peptide produced.

Circulating hepcidin levels are mainly derived from the liver. Hepcidin is also expressed by other cells, such as the heart (8;9), albeit at a much lower level by comparison (10). Locally produced hepcidin affects iron homeostasis in an autocrine fashion, as shown by a downregulation of Fpn-1 expression by hepcidin produced by inflammatory monocytes (11). In vitro, hepcidin reduces Fpn-1 content and iron release in cardiomyocytes (12), suggesting that hepcidin is involved in regulating cardiac iron turn-over. Previous studies show that myocardial ischemia induces cardiac hepcidin expression and consequently enhances ferritin content in cardiomyocytes, this being related to the severity of ischemia (8;9). This suggests that, in addition to systemic regulation, hepcidin expression is also regulated at organ level and may influence cardiac iron content in response to myocardial injury. Indeed, cardiac hepcidin expression is regulated by a number of factors including hypoxia and inflammation (8).

Recently, we developed a rodent model of chronic renocardiac failure by subtotal nephrectomy (SNX) followed by coronary ligation (CL) (13). CL superimposed on SNX leads to more severe heart failure. For the current study, we assessed cardiac and hepatic hepcidin mRNA expression and related regulatory genes as well as ferritin protein expression, an indicator of local iron stores in this model.

The present study investigated the assumption that hepcidin expression in liver and heart is differentially regulated. We hypothesized that cardiac hepcidin is upregulated in response to damage, both in models that induce damage to the myocardium directly (i.e. myocardial infarction (MI) induced by coronary ligation) as well as in models that damage the myocardium indirectly (i.e. chronic kidney disease (CKD) induced by renal ablation). Furthermore, we hypothesized that the induction of hepcidin gene expression is related to the severity of cardiac injury and increases local iron content.

Methods

Study Design

The study protocol was approved by the Ethics Committee on Animal Experiments of the University of Utrecht, Utrecht, the Netherlands. Male inbred Lewis rats (Lew/Crl; 180-200 grams) were purchased from Charles River, Germany, and housed in a climate-controlled facility with a 12:12-hour light:dark cycle. From t= -1 wk to wk 0 a two-stage subtotal nephrectomy by resection or sham operation was performed as described previously (13). Briefly, first the right kidney was removed and one week later the poles of the left kidney were cut off. Adequacy of the SNX procedure was confirmed by an increase in plasma urea. From t=1 wk onward, rats received standard powdered chow supplemented with 6% NaCl until the end of the study. In Lewis rats, high salt intake is required to induce fluid overload and hypertension after SNX (13). At t= 9 wks, rats from both groups were either subjected to CL or sham operation. This resulted in four groups: CON (sham-SNX+sham-CL; n=10); SNX (SNX+sham-CL; n=12); CL (sham-SNX+CL; n=9); and SNX+CL (n=9). Rats were followed up to wk 16. To assess end diastolic volume (EDV), transthoracic echocardiography was performed with a digital ultrasound machine (model Sonos 5500, Philips Research, Eindhoven, The Netherlands) and a 15-MHz linear array transducer (Hewlett Packard, Palo Alto, CA). CL and SNX+CL rats without visible MI on echocardiography and an ejection fraction (EF) ≥ 40% at wk 11 were excluded from the study. EF was calculated from end-diastolic and end-systolic volumes obtained with the area-length calculation, on B-mode cine loops recorded in the parasternal long axis view (13). In wk 16, rats were subjected to isoflurane anaesthesia, euthanized and organs were removed, weighed and processed for histological quantification and determination of mRNA expression. Left ventricular mass index (LVMI) was calculated as percentage of total body weight (BW, g/100 g). Infarct size was measured on photomicrographs of transverse sections of the heart stained with Sirius Red by dividing the length of the infarct scar by the circumference of the total LV section, traced in the midwall using ImageJ software. Fibrosis was measured on photographs of transverse sections (in remote myocardium of hearts with MI) stained with Sirius Red. Photographs were taken using a polarization filter and analysed using Adobe Photoshop and ImageJ software. All measurements were performed by an experienced technician blinded to the group allocation. The study protocol was approved by the Ethics Committee on Animal Experiments of the University of Utrecht, Utrecht, The Netherlands.

Immunohistochemistry

Macrophages were stained with an antibody to ED1 and lymphocytes with an antibody to CD3 as previously described (13). ED1- and CD3 positive cells were counted in the left ventricle. Renal tissue was paraffin-embedded and cut into 3-μ sections. Perls' Prussian Blue staining was

performed to assess iron deposition. Besides Prussian Blue as a histopathology stain, ferritin immunostaining was used in cardiac and liver tissue to detect the presence of iron. Paraffin sections were deparaffinised with xylene and rehydrated with a series of decreasing ethanol concentrations. Next, an antigen retrieval step using citrate was performed. To block endogenous peroxidise activity, the sections were incubated with 1% BSA. The primary antibody (rabbit anti-ferritin, Sigma-Aldrich) was incubated overnight at 4°C in a 1:50 dilution. A polyclonal biotinylated swine anti-rabbit immunoglobulin antibody was used as secondary antibody in a 1:500 dilution for 30 min at RT. Section were subsequently incubated for 30 min with an avidin biotin complex, followed by 3 min of 3.3'-diaminobenzidine (DAB). Quantification of ferritin staining was done by means of calculating the reciprocal intensity using ImageJ software, with an average of 10 images per section(14). In all CL rats immunohistochemistry was evaluated in regions distant from the infarct.

RNA isolation and quantitative polymerase chain reaction

Messenger RNA (mRNA) expression of hepcidin (Hamp; Rn00221783), brain natriuretic peptide (Bnp; Rn00580641), connective tissue growth factor (Ctgf; Rn00573960), ferroportin (Fpn1 = Slc40a1, Rn00591187), heme oxygenase-1 (Ho-1, Rn00561387) and bone morphogenetic protein 6 (Bmp6, Rn 00432095) in cardiac apical tissue (distant from the infarct in CL rats), and of hepcidin and C/ebp-α (Rn00560963) in liver tissue was assessed by qPCR as described previously (15).

For the real-time PCR using the BIOMARK device (Fluidigm, San Francisco, CA), RNA was purified from 2ug total RNA samples using the RNeasy Micro kit (Qiagen, Toronto, Canada) and genomic DNA was removed by RNase-free DNase (Qiagen). Quality and quantity of all RNA samples was using a Bio-Analyzer (Agilent, Santa Clara, CA). Reverse transcription was carried out on 200-500ng total RNA per sample using SuperScript® II Reverse Transcriptase and random primers (Thermo Fisher Scientific). The following TaqMan primers were used (all from Thermo Fisher Scientific): Calnexin (Rn01459976_m1), Actin-β (Rn00667869_m1), IL1β (Rn00580432_m1), IL6 (Rn01410330_m1), caspase 3 (Rn00563902_m1), TNFR-1 (Rn01492348_m1), TLR4 (Rn00569848_m1), Cebpa (CCAAT/enhancer binding protein (C/EBP), alpha) (Rn00560963_s1), TNF α(Rn01525860_g1) and TLR2 (Rn01769726_m1). Then, each primer was mixed with DNA suspension buffer (TEKNOVA, Hollister, USA and each cDNA sample was added. Following addition of preamp master mix (Fluidigm), 14 circles of pre-amplification were performed on a thermocycler (Bio-Rad, Hercules, CA). Then, the real-time PCR of each sample in duplicate was performed using the 10 primers in duplicate using a 48*48 IFC and the Fluidigm Biomark HD instrument. The data were collected and analyzed using the Fluidigm Data Collection system, and

Ct values were calculated from the software of BioMark Real-Time PCR Analysis. For all samples at least 4 successful reactions were obtained.

Cycle time (Ct) values for all genes were normalized to mean Ct-values of Calnexin (Canx; Rn00596877) and β-actin (Actb; Rn00667869), which we previously determined to be the two most stable housekeeping genes across all groups in both organs using the geNorm program (http://medgen.ugent.be/jvdesomp/genorm/). This produced ΔC_t values. Gene expression relative to CON was calculated by subtracting the ΔC_t-values from the mean ΔC_t of the CON group, producing the $\Delta\Delta C_t$ values. Statistical analysis was performed on $\Delta\Delta C_t$ values, and results were graphed as fold changes compared to CON ($2\Delta\Delta^{Ct}$).

Statistical analysis

Data were analysed and graphed using SigmaPlot 12.3 (Systat Software, San Jose, CA). Data that were not normally distributed were log-transformed to achieve normality. Statistical analysis was performed by 2-way ANOVA with the Student-Newman-Keuls post hoc test. Statistical significance was reached with P values below 0.05. Univariate linear regression was used to test correlations.

Results

General results

Longitudinal functional and structural characterization of this model has been published elsewhere (13). At week 15, rats with SNX had lower body weight than rats without SNX, which was not affected by subsequent CL (Table 1). Subtotal nephrectomy caused reduced creatinine clearance (P< 0.001 vs. CON), that was not significantly affected by CL in either CL or SNX+CL rats. Given the differences in body weight, cardiac parameters are expressed per 100 gram body weight, where applicable. Cardiac index was lower in SNX+CL than in SNX (P< 0.01) and tended to be lower in SNX+CL vs. CL (P= 0.065). LV mass index (LVMI) increased in SNX rats (P< 0.001 vs. CON) but decreased in SNX+CL, while EDV increased only in CL (P< 0.01 vs. CON) and SNX+CL rats (P< 0.01 vs SNX).

Table 1. Biometric and terminal inflammation variables in the heart at week 15 [13]

	Con (n=10)	CON+CL (n=9)	SNX (n=10)	SNX+CL (n=7)
Body weight (g)	424 ± 8	426 ± 9	378 ± 11***	361 ± 14 ###
Haematocrit (ml/ml)	0.47 ± 0.01	0.46 ± 0.01	0.42 ± 0.01**	0.43 ± 0.02
Kidney:				
Creatinine clearance (ml/min/100 g BW)	0.74 ± 0.06	0.67 ± 0.09	0.30 ± 0.03***	0.27 ± 0.04###
Heart:				
Cardiac index (ml/min/100 g BW)	30 ± 2	17 ± 1 ***	29 ± 2	14 ± 1$$$
LV mass index (g/100 g BW)	0.19 (0.19-0.20, n=8)	0.21 (0.20-0.23, n=5)	0.35 (0.32-0.39, n=7)***	0.29 (0.26-0.30, n=6)$$$***
EDV	0.54 ±0.03, n=8	0.83 ± 0.11, n=5***	0.70 ± 0.06, n=6	0.97 ± 0.05, n=6$$
CD $^{3+}$ cells in myocardium (per HPF)	1.2 (0.1-2.8)	0.5 (0-1.4)	0.5 (0-1.9)	0.5 (0.2-1.6)
ED^{1+} cells in myocardium (per HPF)	11 (5-15)	16 (12-26)**	11 (7-22)	16 (5-25)

Data as mean ± SEM and as median (range); *n,* no. of rats, BW = body weight, HPF= high-power field. ** P< 0.01 vs CON, *** P< 0.001 vs CON, # P< 0.05 vs CL, ### P< 0.001 vs CL, $$ P< 0.01 vs SNX, $$$ P< 0.001 vs SNX. These data were published previously [13].

Immunohistochemistry

The number of inflammatory CD3-positive lymphocytes (T-cells) in the remote myocardium was not different between groups (table 1). Inflammatory ED1-positive cells (monocytes and macrophages) were increased in the remote myocardium after CL compared to CON (P< 0.01), irrespective of the presence of SNX.

Hepcidin mRNA expression in heart and liver

To investigate the differential regulation of hepcidin, we assessed hepcidin, i.e. Hamp, gene expression in both heart and liver tissue. Compared to CON hepcidin mRNA expression in cardiac tissue was increased two-fold in CL (P= 0.03) and three-fold in SNX (P= 0.01, figure 1). In contrast, hepatic mRNA expression of hepcidin was unaffected by SNX and CL alone, while it was significantly decreased (50%, P < 0.05) in SNX+CL compared to both SNX and CL alone.

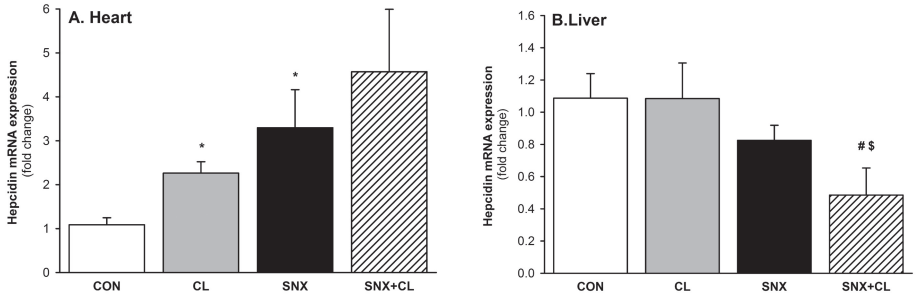


Figure 1. Hepcidin mRNA expression in (A) heart and (B) liver. ΔΔCt: Ct values of target gene normalized to mean Ct values of housekeeping genes and the mean Ct value of the CON group. Mean ± SEM, * P< 0.05 vs CON; # P< 0.05 vs CL; $ P< 0.05 vs SNX. N heart: CON 9, CL 6, SNX 7, SNX+CL 6. N liver: CON 8, CL 7, SNX 8, SNX+CL 4.

Iron content in heart and liver

We used Prussian Blue staining to localize iron storage in heart tissue. In the remote myocardium of CL rats practically no iron was observed, similar to hearts of non-infarcted (SNX or CON) rats. To extend our observations, we also performed ferritin immunohistochemistry. In line with the Prussian Blue staining, no differences in ferritin content in the remote myocardium of CL and SNX+ CL rats, as compared to non-infarcted rats were observed (figure 2 A). In liver tissue, there were also no differences seen in ferritin content in CL and SNX+CL rats compared to SNX or CON (figure 2B).

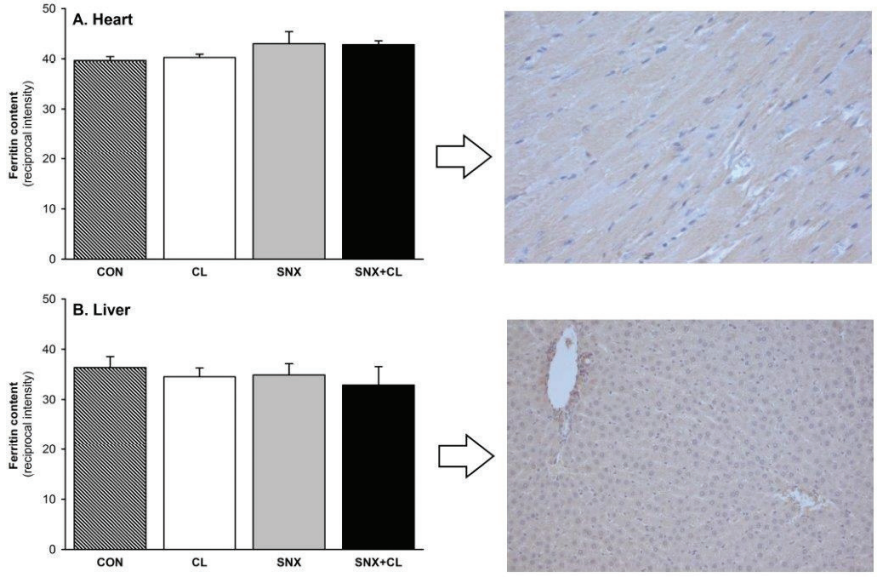


Figure 2. Quantification of ferritin staining assessed by immunohistochemistry and representative images of ferritin in (A) cardiac tissue and (B) liver tissue. N heart: CON 5, CL 4, SNX 4, SNX+CL 4. N liver: CON 4, CL 4, SNX 5, SNX+CL 3.

Cytokine expression in cardiac tissue

To confirm that inflammation was related to cardiac hepcidin expression, we measured cardiac cytokine gene expressions involved in inflammation and apoptosis. Cardiac gene expression of IL-6, IL-1β, caspase3 and calnexin tended to increase in SNX and CL, but the only gene expression reaching statistical significance was IL-1β. Both SNX and CL independently increased cardiac IL-1β gene expression (increase six-fold, P< 0.001 and increase eight-fold, P< 0.001 respectively, figure 3). The combination of both SNX and CL did not further increase cardiac IL-1β gene expression.

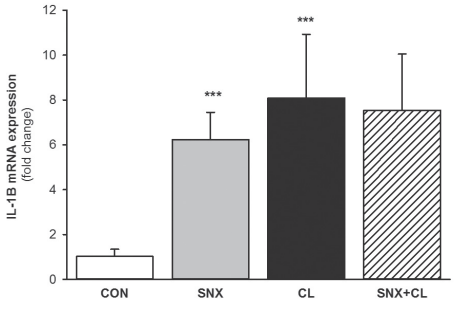


Figure 3. IL-1β expression in cardiac tissue, assessed by qPCR. ΔΔCt: Ct values of target gene normalized to mean Ct values of housekeeping genes and the mean Ct value of the CON group. Mean ± SEM, *** P< 0.001 vs CON. N: CON 8, CL 5, SNX 12, SNX+CL 6.

Bmp6 mRNA expression in cardiac tissue

The extracellular signalling molecule Bmp6 enhances transcription of the hepatic hepcidin gene in mice (16). Cardiac Bmp6 mRNA expression level followed the same pattern as cardiac hepcidin mRNA expression. Bmp6 was significantly increased in SNX rats (P< 0.01), while the largest increase was seen in SNX+CL rats (figure 4).

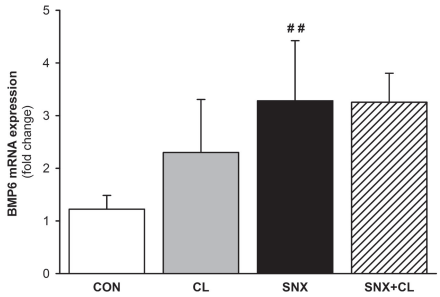


Figure 4. Bone Morphogenetic Protein 6 expression in cardiac tissue, assessed by qPCR. ΔΔCt: Ct values of target gene normalized to mean Ct values of housekeeping genes and the mean Ct value of the CON group. Mean ± SEM, ## P< 0.01 vs CL. N: CON 10, CL 6, SNX 7, SNX+CL 6.

Cardiac BNP and CTGF mRNA expression

In order to investigate the degree of damage in cardiac tissue, we measured brain natriuretic peptide (Bnp) and connective tissue growth factor (Ctgf) mRNA expression levels as markers of injury. Cardiac mRNA expression levels of Bnp were increased 2.2-fold and 1.8-fold in SNX and in CL rats, respectively with a 2.8-fold increase in SNX+CL as reported (13). Cardiac mRNA expression levels of Ctgf were increased 45-fold and 18-fold in SNX and in CL rats, respectively with a 61-fold increase in SNX+CL as reported (13).

Heme Oxygenase-1 and ferroportin-1 mRNA expression in cardiac tissue

To get more insight in regulation of iron in the heart, we assessed mRNA expression levels of other proteins involved in iron metabolism such as heme oxygenase-1 (Ho-1) and ferroportin-1 (Fpn-1) (17). It is well known that Ho-1 acts as an anti-oxidant against oxidative injury which could inhibit the production of reactive oxygen species and reduce oxidative damage (18;19). Cardiac mRNA expression of Ho-1 was significantly increased in SNX (P< 0.01 vs. CON). Ho-1 expression level in SNX+CL tended to be lower than SNX and resembled the level of CL (Figure 5A). Cardiac mRNA expression of Fpt1 showed the same pattern as Ho-1, with a stepwise increase in CL and SNX. Similar to Ho-1, Fpn-1 gene expression in SNX+CL was less than in SNX and similar to CL. Two-way ANOVA showed significant interaction between SNX and CL (Figure 5B, P= 0.002).

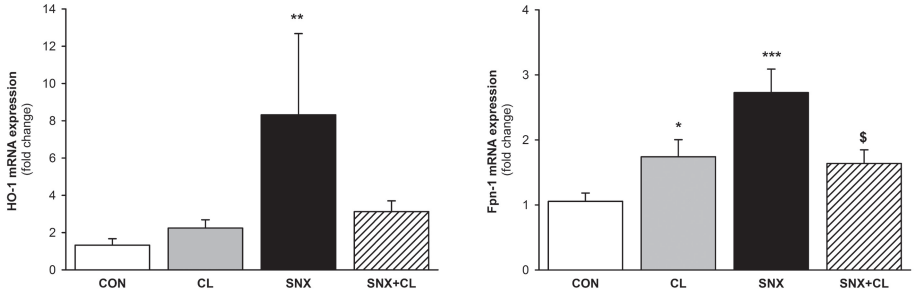


Figure 5. Heme Oxygenase-1 expression (A) and ferroportin-1 expression (B) in cardiac tissue, assessed by qPCR.ΔΔCt: Ct values of target gene normalized to mean Ct values of housekeeping genes and the mean Ct value of the CON group. Mean ± SEM, * P< 0.05 vs CON, ** P< 0.01 vs CON, *** P< 0.001 vs CON, $ P< 0.05 vs SNX. N: CON 10, CL 6, SNX 7, SNX+CL 6.

Hepatic mRNA expression of C/EBP α

Since CCAAT/enhancer-binding protein α (C/ebp α) plays a key role in regulation of Hamp gene expression in the liver (20), we expected the same pattern for hepatic C/ebp α mRNA expression as for hepatic hepcidin mRNA expression. Indeed, hepatic mRNA expression of C/ebp α was decreased progressively across groups with the largest decrease in SNX+CL (P< 0.05 vs. CL and P< 0.05 vs. SNX, figure 6).

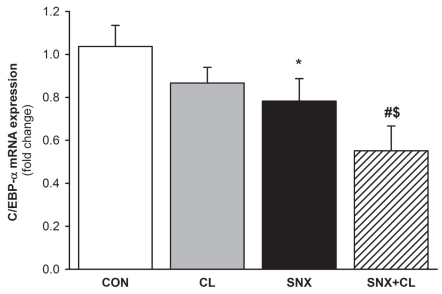


Figure 6. Hepatic expression of c/EBP α across groups. ΔΔCt values of target gene normalized to mean Ct values of housekeeping genes and the mean Ct value of the CON group. Mean ± SEM, * P< 0.05 vs CON, # P< 0.05 vs CL, $ P< 0.05 vs SNX. N: CON 9, CL 7, SNX 9, SNX+CL 4.

Correlations

In order to investigate possible factors influencing cardiac and hepatic hepcidin expression, correlations were analysed. Cardiac hepcidin mRNA expression correlated linearly with Bnp mRNA expression (β= 0.73, P< 0.001, figure 7A) and Ctgf mRNA expression (β= 0.43, P= 0.02, figure 7B) but not with Bmp6 mRNA expression (β= 0.23, P= 0.25, figure 7C). Cardiac hepcidin mRNA expression strongly correlated with pro-inflammatory cytokine IL-1β (β= 0.47, P= 0.02, figure 7D). However, cardiac hepcidin mRNA expression did not correlate with cardiac ferritin immunostaining (β= 0.16, P= 0.54).

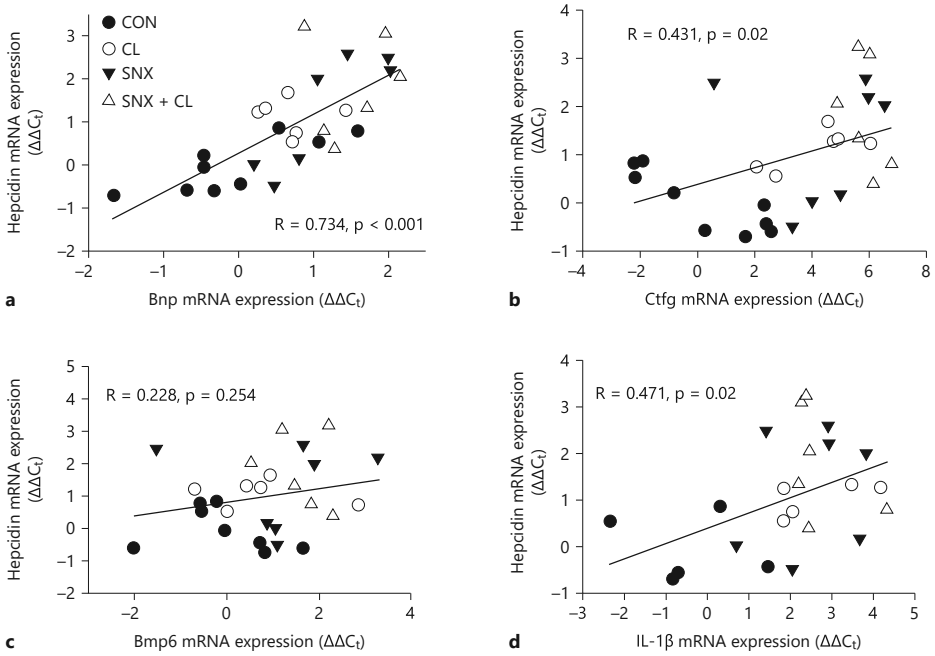


Figure 7. Correlations between cardiac expression of hepcidin and Bnp (a), cardiac hepcidin and Ctgf (b), cardiac hepcidin and Bmp6 (c) and cardiac hepcidin and IL-1β(d). ΔΔCt values of target gene normalized to mean Ct values of housekeeping genes and the mean Ct value of the CON group

Hepatic hepcidin mRNA expression correlated with hepatic mRNA expression of the transcription factor C/ebp α (β= 0.68, P< 0.001, Figure 8) but not with ferritin staining (β= 0.14, P= 0.64).

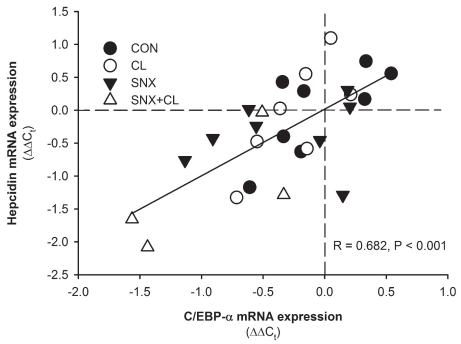


Figure 8. Correlation between hepatic gene expression of c/EBP α and hepatic hepcidin. ΔΔCt values of target gene normalized to mean Ct values of housekeeping genes and the mean Ct value of the CON group.

Both left ventricular mass index (LVMI) and end diastolic volume (EDV) correlated significantly with cardiac hepcidin mRNA expression (β= 0.53, P< 0.01 and β= 0.62, P< 0.001 resp., figure 9 A and B). The correlation between cardiac hepcidin mRNA expression and percentage of Sirius Red stained area as measure of cardiac fibrosis was not significant (β= 0.35, P= 0.1, figure 9 C).

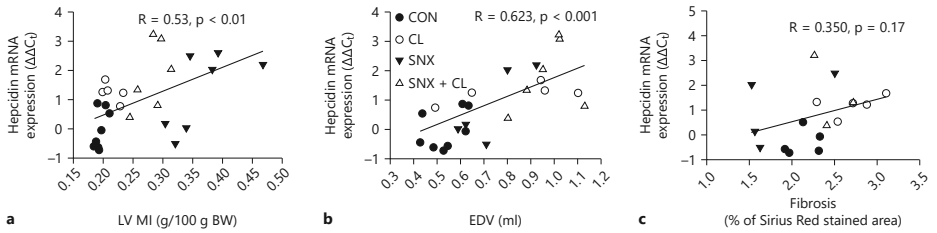


Figure 9. Correlation between cardiac hepcidin and LV mass index (A), cardiac hepcidin and EDV (B) and cardiac hepcidin and fibrosis (C). ΔΔCt values of target gene normalized to mean Ct values of housekeeping genes and the mean Ct value of the CON group.

Discussion

The aim of this study was to investigate regulation of hepcidin at tissue level in experimental chronic renocardiac failure. The main finding of this study was that cardiac hepcidin (Hamp) gene expression is significantly induced in both local (CL inducing MI) and remote (SNX inducing CKD) injury. Conversely, liver hepcidin gene expression was decreased. Cardiac iron content in non-infarcted tissue remained unchanged in all experimental groups. Thus, cardiac hepcidin expression was increased in response to injury, but no evidence of an association with local iron accumulation was observed.

The increased cardiac expression of hepcidin is in agreement with findings in other models with cardiac injury and myocarditis (8;9;21). In rats hypoxia and inflammation were shown to upregulate hepcidin expression (8). In agreement with this finding hepcidin expression was found to be induced in rat hearts with myocardial infarction (MI) and myocarditis, as well as in human hearts with myocarditis (9). Furthermore, increased expression of hepcidin was found in the hearts of rats with CKD, which was associated with levels of iron deficiency and anemia (21).

We assessed the mRNA expression of the cardiac damage markers Bnp and Ctgf in myocardial tissue. Bnp is a well-known marker of cardiac dysfunction and has an anti-fibrotic function (22), Ctgf is a profibrotic cytokine of the Ccn (Cyr61, Ctgf, and Nov) family (23) and is suggested to serve as a diagnostic marker of cardiac dysfunction (24;25). As shown, there is a progressive increase of Bnp and Ctgf gene expression in the CL, SNX and SNX+CL group. Cardiac hepcidin expression

in the SNX+CL group was significantly and positively correlated with cardiac injury based on Bnp and Ctgf but not with ferritin staining. Based on these findings we postulate that cardiac hepcidin mRNA expression can be induced by local or remote injury and is not necessarily dependent on local iron stores. In the remote myocardium, even though there is no evidence of direct myocardial damage and iron accumulation, hepcidin expression is upregulated.

To confirm our hypothesis that local injury in the heart affects cardiac hepcidin gene expression, we measured the expression of several inflammatory cytokine gene expressions in the heart. Although the cardiac gene expression of IL-6, IL-1β, caspase3 and calnexin tended to increase in SNX and CL, suggesting inflammation and apoptosis, the only gene expression that reached statistical significance is IL-1β. High blood levels of IL-1β have been observed after myocardial infarction (26) and it is demonstrated that IL-1β induce myocyte hypertrophy (27). Interestingly, IL-1β also increased in the SNX group. The pathophysiological mechanism of renal failure and increased cardiac IL-1β expression is not elucidated. In experimental models of nephrotic syndrome, glomerular expression of IL-1β is demonstrated (28), probably due to systemic inflammation. Moreira-Rodrigues et al showed elevated expression of cardiac IL-1β expression in a rat model with nephrotic syndrome (29) and also in rats with spontaneous hypertension high levels of IL-1β were measured in the kidneys (30) The strong correlation between cardiac hepcidin gene expression and cardiac IL-1β supports the thought that cardiac hepcidin regulation is driven by inflammation instead of iron.

Several authors speculate that hepcidin plays an important protective role against extracellular free radical formation since it decreases the presence of the transmembrane iron exporter ferroportin and thus inhibits cellular iron export (8;9). Due to the large amount of iron in cardiomyocytes, destruction of these cells could result in high iron concentrations in the extracellular space. It is well known that free iron generates damaging reactive oxygen species (ROS) and causes various types of injury (31), including myocardial fibrosis. Intracellular iron is stored in ferritin, an important intracellular iron sequestering and storage protein that plays a cytoprotective role against free radical formation by controlling the free cytosolic iron concentration (32). It could be that during myocardial damage, cardiac hepcidin is upregulated to prevent iron release from the intact cells. However, in contrast to this possible mechanism, in our study cardiac ferritin content was not related to hepcidin expression. Our data suggest that hepcidin gene expression is influenced by local injury not related to local iron turn over; there is enhanced cardiac hepcidin mRNA expression, in the absence of changed cardiac ferritin content. Besides, liver expression of Bmp6 is transcriptionally regulated by iron: iron overload increases Bmp 6 expression, iron deficiency represses Bmp6 expression (33). However, in our study, cardiac BMP6 mRNA expression

was significantly increased in SNX and SNX+CL rats, but no differences in iron content in the remote myocardium was observed. This could implicate that in the heart, it is not iron, but other stimuli such as injury, which stimulate Bmp6 expression. Bmp6 expression could be part of a compensatory mechanism in the heart to stimulate cardiomyocyte hypertrophy (34). Korf-Klingebiel et al found that recombinant Bmp6 (even in low concentrations) resulted in a robust increase in cell size and protein synthesis in neonatal and adult cardiomyocytes, respectively.

In contrast to hepcidin and the damage markers Bnp and Ctgf, and contrary to our expectations, Fpn-1 and Ho-1 mRNA expression showed a different pattern. For Fpn-1 and Ho-1 there was an interaction between SNX and CL, in that the presence of both eliminated the SNX effect. The basis for this observation is not clear. Although it is widely assumed that hepcidin induces ferroportin internalization and degradation, this relation is not as clear as it seems. In monocytes of HD patients no correlation between serum hepcidin and ferroportin was found (35). This suggests that factors other than hepcidin also affect ferroportin levels and internalization and that degradation of ferroportin can take place in a hepcidin-independent manner. The fact that this relation is absent in cell types in which hepcidin is thought to play its classical role (ferroportin internalization and degradation), supports our result regarding the lack of such a relation at the mRNA level in the heart in our cardiorenal syndrome model.

Unexpectedly, combined SNX and CL showed a decrease in hepatic hepcidin mRNA expression. Both cardiac and renal failure is associated with an increase of inflammatory cytokines, which could have resulted in an upregulation of hepatic hepcidin gene expression (36). However, several studies reported low serum hepcidin levels in patients with chronic heart failure and anemia (37;38). One possible explanation is that iron deficiency, even in the presence of increased cytokines, leads to diminished levels of hepcidin in conditions of heart failure and anemia. Although in our rodent model only mild anemia was present, anemia and increased erythropoiesis (ie, production of red blood cells) could down regulate the synthesis of hepcidin mRNA (39). Unfortunately, we did not measure circulating inflammatory cytokines and iron markers to confirm this hypothesis.

In agreement with this finding, we showed a correlation between mRNA expressions of C/ebp α and hepcidin in the liver, in the sense that both were reduced. C/ebp α is a transcription factor in the liver that has a positive effect on Hamp promotor activity (20). Alcoholic and viral liver cell damage both reduce hepcidin expression, a reduction which is mediated by reactive oxygen species via C/ebp α (40;41). Reduction in hepatic C/ebp α mRNA expression in renocardiac failure may associate with hepatic oxidative stress (42).

LVMI increased in SNX rats and decreased in SNX+CL rats, most probably due to the loss of cardiomyocytes after cardiac injury. As a measure of cardiac preload and increased filling pressures, EDV increased in CL and SNX+CL rats. These results are in line with previous findings that left ventricular mass increases during renal and cardiac failure, the predominant pattern being eccentric left ventricular hypertrophy (43;44). In accordance with previous research (45), the significant correlation between cardiac hepcidin mRNA expression and LVMI and EDV suggests that hepcidin plays a role in the hypertrophic response in cardiomyocytes. Once again, this emphasizes the role of hepcidin in cardiac damage.

Given the fact that liver hepcidin is only regulated at the transcriptional level, we used HAMP mRNA levels to detect cardiac hepcidin. Accordingly, we studied the stimulatory effect of cardiac injury on cardiac hepcidin expression rather than the reverse.

In conclusion, cardiac expression of hepcidin is differentially regulated in this rat model of renocardiac failure from its primary source of production, the liver. Moreover, our data suggest a role for injury rather than iron as a driving force for cardiac hepcidin expression in experimental renocardiac failure. Future animal and cell experiments should be developed to study the mechanisms of systemic and local hepcidin production and action in different tissues. Elucidating the pathophysiological mechanisms of local hepcidin regulation in patients with CKD and/or heart failure may help designing new therapeutic approaches.

References

1. Macdougall IC, Canaud B, de Francisco AL, Filippatos G, Ponikowski P, Silverberg D, van Veldhuisen DJ, Anker SD: Beyond the cardiorenal anaemia syndrome: recognizing the role of iron deficiency. Eur J Heart Fail 2012;14:882-886.

2. Anker SD, Comin CJ, Filippatos G, Willenheimer R, Dickstein K, Drexler H, Luscher TF, Bart B, Banasiak W, Niegowska J, Kirwan BA, Mori C, von Eisenhart RB, Pocock SJ, Poole-Wilson PA, Ponikowski P: Ferric carboxymaltose in patients with heart failure and iron deficiency. N Engl J Med 2009;361:2436-2448.

3. Jankowska EA, Rozentryt P, Witkowska A, Nowak J, Hartmann O, Ponikowska B, Borodulin-Nadzieja L, Banasiak W, Polonski L, Filippatos G, McMurray JJ, Anker SD, Ponikowski P: Iron deficiency: an ominous sign in patients with systolic chronic heart failure. Eur Heart J 2010;31:1872-1880.

4. Gujja P, Rosing DR, Tripodi DJ, Shizukuda Y: Iron overload cardiomyopathy: better understanding of an increasing disorder. J Am Coll Cardiol 2010;56:1001-1012.

5. Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T, Kaplan J: Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 2004;306:2090-2093.

6. Meynard D, Babitt JL, Lin HY: The liver: conductor of systemic iron balance. Blood 2014;123:168-176.

7. Lin L, Valore EV, Nemeth E, Goodnough JB, Gabayan V, Ganz T: Iron transferrin regulates hepcidin synthesis in primary hepatocyte culture through hemojuvelin and BMP2/4. Blood 2007;110:2182-2189.

8. Merle U, Fein E, Gehrke SG, Stremmel W, Kulaksiz H: The iron regulatory peptide hepcidin is expressed in the heart and regulated by hypoxia and inflammation. Endocrinology 2007;148:2663-2668.

9. Isoda M, Hanawa H, Watanabe R, Yoshida T, Toba K, Yoshida K, Kojima M, Otaki K, Hao K, Ding L, Tanaka K, Takayama T, Kato K, Okura Y, Kodama M, Ota Y, Hayashi J, Aizawa Y: Expression of the peptide hormone hepcidin increases in cardiomyocytes under myocarditis and myocardial infarction. J Nutr Biochem 2010;21:749-756.

10. Kroot JJ, Tjalsma H, Fleming RE, Swinkels DW: Hepcidin in human iron disorders: diagnostic implications. Clin Chem 2011;57:1650-1669.

11. Theurl I, Theurl M, Seifert M, Mair S, Nairz M, Rumpold H, Zoller H, Bellmann-Weiler R, Niederegger H, Talasz H, Weiss G: Autocrine formation of hepcidin induces iron retention in human monocytes. Blood 2008;111:2392-2399.

12. Ge XH, Wang Q, Qian ZM, Zhu L, Du F, Yung WH, Yang L, Ke Y: The iron regulatory hormone hepcidin reduces ferroportin 1 content and iron release in H9C2 cardiomyocytes. J Nutr Biochem 2009;20:860-865.

13. Bongartz LG, Joles JA, Verhaar MC, Cramer MJ, Goldschmeding R, Tilburgs C, Gaillard CA, Doevendans PA, Braam B: Subtotal nephrectomy plus coronary ligation leads to more pronounced damage in both organs than either nephrectomy or coronary ligation. Am J Physiol Heart Circ Physiol 2012;302:H845-H854.

14. Nguyen DH, Zhou T, Shu J, Mao J-H: Quantifying chromogen intensity in immunohistochemistry via reciprocal intensity. Cancer InCytes 2013;2.

15. Wesseling S, Koeners MP, Kantouh F, Joles JA, Braam B: Consequences of perinatal treatment with L-arginine and antioxidants for the renal transcriptome in spontaneously hypertensive rats. Pflugers Arch 2009;458:513-524.

16. Andriopoulos B, Jr., Corradini E, Xia Y, Faasse SA, Chen S, Grgurevic L, Knutson MD, Pietrangelo A, Vukicevic S, Lin HY, Babitt JL: BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism. Nat Genet 2009;41:482-487.

17. Ganz T: Systemic iron homeostasis. Physiol Rev 2013;93:1721-1741.

18. Ryu MJ, Kang KA, Piao MJ, Kim KC, Zheng J, Yao CW, Cha JW, Chung HS, Kim SC, Jung E, Park D, Chae S, Hyun JW: 7,8-Dihydroxyflavone protects human keratinocytes against oxidative stress-induced cell damage via the ERK and PI3K/Akt-mediated Nrf2/HO-1 signaling pathways. Int J Mol Med 2014;33:964-970.

19. Liu K, Chen HL, Huang H, Jing H, Dong GH, Wu HW, You QS: Curcumin attenuates cardiopulmonary bypass-induced lung oxidative damage in rats. J Cardiovasc Pharmacol Ther 2012;17:395-402.

20. Courselaud B, Pigeon C, Inoue Y, Inoue J, Gonzalez FJ, Leroyer P, Gilot D, Boudjema K, Guguen-Guillouzo C, Brissot P, Loreal O, Ilyin G: C/EBPalpha regulates hepatic transcription of hepcidin, an antimicrobial peptide and regulator of iron metabolism. Cross-talk between C/EBP pathway and iron metabolism. J Biol Chem 2002;277:41163-41170.

21. Toblli JE, Cao G, Rivas C, Kulaksiz H: Heart and iron deficiency anaemia in rats with renal insufficiency: the role of hepcidin. Nephrology (Carlton) 2008;13:636-645.

22. Nishikimi T, Maeda N, Matsuoka H: The role of natriuretic peptides in cardioprotection. Cardiovasc Res 2006;69:318-328.

23. Blom IE, Goldschmeding R, Leask A: Gene regulation of connective tissue growth factor: new targets for antifibrotic therapy? Matrix Biol 2002;21:473-482.

24. Koitabashi N, Arai M, Niwano K, Watanabe A, Endoh M, Suguta M, Yokoyama T, Tada H, Toyama T, Adachi H, Naito S, Oshima S, Nishida T, Kubota S, Takigawa M, Kurabayashi M: Plasma connective tissue growth factor is a novel potential biomarker of cardiac dysfunction in patients with chronic heart failure. Eur J Heart Fail 2008;10:373-379.

25. Koitabashi N, Arai M, Kogure S, Niwano K, Watanabe A, Aoki Y, Maeno T, Nishida T, Kubota S, Takigawa M, Kurabayashi M: Increased connective tissue growth factor relative to brain natriuretic peptide as a determinant of myocardial fibrosis. Hypertension 2007;49:1120-1127.

26. Guillen I, Blanes M, Gomez-Lechon MJ, Castell JV: Cytokine signaling during myocardial infarction: sequential appearance of IL-1 beta and IL-6. Am J Physiol 1995;269:R229-R235.

27. Neumann DA, Lane JR, Allen GS, Herskowitz A, Rose NR: Viral myocarditis leading to cardiomyopathy: do cytokines contribute to pathogenesis? Clin Immunol Immunopathol 1993;68:181-190.

28. Gomez-Chiarri M, Ortiz A, Lerma JL, Lopez-Armada MJ, Mampaso F, Gonzalez E, Egido J: Involvement of tumor necrosis factor and platelet-activating factor in the pathogenesis of experimental nephrosis in rats. Lab Invest 1994;70:449-459.

29. Moreira-Rodrigues M, Roncon-Albuquerque R, Jr., Henriques-Coelho T, Lourenco AP, Sampaio-Maia B, Santos J, Pestana M, Leite-Moreira AF: Cardiac remodeling and dysfunction in nephrotic syndrome. Kidney Int 2007;71:1240-1248.

30. Sun L, Gao YH, Tian DK, Zheng JP, Zhu CY, Ke Y, Bian K: Inflammation of different tissues in spontaneously hypertensive rats. Sheng Li Xue Bao 2006;58:318-323.

31. Mladenka P, Simunek T, Hubl M, Hrdina R: The role of reactive oxygen and nitrogen species in cellular iron metabolism. Free Radic Res 2006;40:263-272.

32. Balla G, Jacob HS, Balla J, Rosenberg M, Nath K, Apple F, Eaton JW, Vercellotti GM: Ferritin: a cytoprotective antioxidant strategem of endothelium. J Biol Chem 1992;267:18148-18153.

33. Kautz L, Meynard D, Monnier A, Darnaud V, Bouvet R, Wang RH, Deng C, Vaulont S, Mosser J, Coppin H, Roth MP: Iron regulates phosphorylation of Smad1/5/8 and gene expression of Bmp6, Smad7, Id1, and Atoh8 in the mouse liver. Blood 2008;112:1503-1509.

34. Korf-Klingebiel M, Kempf T, Schluter KD, Willenbockel C, Brod T, Heineke J, Schmidt VJ, Jantzen F, Brandes RP, Sugden PH, Drexler H, Molkentin JD, Wollert KC: Conditional transgenic expression of fibroblast growth factor 9 in the adult mouse heart reduces heart failure mortality after myocardial infarction. Circulation 2011;123:504-514.

35. Eleftheriadis T, Pissas G, Remoundou M, Filippidis G, Antoniadi G, Oustampasidou N, Liakopoulos V, Stefanidis I: Ferroportin in monocytes of hemodialysis patients and its associations with hepcidin, inflammation, markers of iron status and resistance to erythropoietin. Int Urol Nephrol 2014;46:161-167.

36. Tsuchiya K, Nitta K: Hepcidin is a potential regulator of iron status in chronic kidney disease. Ther Apher Dial 2013;17:1-8.

37. Divakaran V, Mehta S, Yao D, Hassan S, Simpson S, Wiegerinck E, Swinkels DW, Mann DL, Afshar-Kharghan V: Hepcidin in anemia of chronic heart failure. Am J Hematol 2011;86:107-109.

38. Matsumoto M, Tsujino T, Lee-Kawabata M, Naito Y, Akahori H, Sakoda T, Ohyanagi M, Tomosugi N, Masuyama T: Iron regulatory hormone hepcidin decreases in chronic heart failure patients with anemia. Circ J 2010;74:301-306.

39. Kautz L, Jung G, Valore EV, Rivella S, Nemeth E, Ganz T: Identification of erythroferrone as an erythroid regulator of iron metabolism. Nat Genet 2014;46:678-684.

40. Harrison-Findik DD, Schafer D, Klein E, Timchenko NA, Kulaksiz H, Clemens D, Fein E, Andriopoulos B, Pantopoulos K, Gollan J: Alcohol metabolism-mediated oxidative stress down-regulates hepcidin transcription and leads to increased duodenal iron transporter expression. J Biol Chem 2006;281:22974-22982.

41. Harrison-Findik DD: Is the iron regulatory hormone hepcidin a risk factor for alcoholic liver disease? World J Gastroenterol 2009;15:1186-1193.

42. van Swelm RP, Laarakkers CM, Blous L, Peters JG, Blaney Davidson EN, van der Kraan PM, Swinkels DW, Masereeuw R, Russel FG: Acute acetaminophen intoxication leads to hepatic iron loading by decreased hepcidin synthesis. Toxicol Sci 2012;129:225-233.

43. Stewart GA, Gansevoort RT, Mark PB, Rooney E, McDonagh TA, Dargie HJ, Stuart R, Rodger C, Jardine AG: Electrocardiographic abnormalities and uremic cardiomyopathy. Kidney Int 2005;67:217-226.

44. Bongartz LG, Soni S, Cramer MJ, Steendijk P, Gaillard CA, Verhaar MC, Doevendans PA, van Veen TA, Joles JA, Braam B: Neuronal nitric oxide synthase-dependent amelioration of diastolic dysfunction in rats with chronic renocardiac syndrome. Cardiorenal Med 2015;5:69-78.

45. Naito Y, Hosokawa M, Sawada H, Oboshi M, Iwasaku T, Okuhara Y, Morisawa D, Eguchi A, Hirotani S, Ohyanagi M, Tsujino T, Masuyama T: Hepcidin is increased in the hypertrophied heart of Dahl salt-sensitive rats. Int J Cardiol 2014;172:e45-e47.

Chapter 5

VITAMIN D AND ANEMIA IN CHRONIC KIDNEY DISEASE

Fenna van Breda and Marc G. Vervloet

Amsterdam UMC, Vrije Universiteit Amsterdam, Nephrology, Amsterdam Cardiovascular sciences, Amsterdam, Netherlands

Vitamin D in Chronic Kidney Disease, chapter 23
Editors: Pablo A. Urena Torres, Mario Cozzolino and Marc G. Vervloet
Springer International Publishing Switzerland 2016

Abstract:

A considerable proportion of patients with chronic kidney disease develop anemia. Several factors are known to contribute to this renal anemia, like EPO deficiency, EPO hyporesponsiveness and functional iron deficiency due to increasing concentrations of hepcidin. Recent studies showing an association in abnormalities of the vitamin D system with low hemoglobin (Hb) levels and erythropoietin stimulating agent (ESA) resistance suggest cross-talk between the vitamin D system and erythropoiesis. The administration of either inactive or active vitamin D has been associated with an improvement of anemia and reduction in EPO hyporesponsiveness. Potential links between the vitamin D system and erythropoiesis are described in this chapter.

Definition and prevalence of anemia

Anemia of chronic kidney disease (CKD) is a common complication among patients with CKD. There is much variability in the hemoglobin (Hb) threshold used to define anemia. According to the most recent definition in the Kidney Disease: Improving Global Outcomes (KDIGO) guidelines, anemia is diagnosed when there is a Hb concentration <13.0 g/dL for adult males and postmenopausal women and an Hb <12.0 g/dL for premenopausal women. A large U.S. survey observed Hb levels < 12 g/dL in more than one in four with relative mild CKD (stage 1 and 2), increasing to more than half of those with severe CKD (stage 4) (1). The prevalence of anemia in patients with CKD is a contributing factor in many symptoms associated with reduced kidney function, including tiredness, fatigue, reduced exercise tolerance and dyspnea (table 1). Anemia has consistently been associated with cardiovascular consequences like left ventricular hypertrophy (LVH) and left ventricular dysfunction (2) and with increased risk of morbidity and mortality due to cardiac disease and stroke (3;4). However, a definite cause-effect relationship has not been proven, so these associations may reflect confounding underlying comorbid conditions and severity of illness that contribute to both the severity of anemia and poor outcomes. This chapter will focus on the different causes of renal anemia and especially on the role of vitamin D in this common complication of patients with CKD.

Table 1: *Symptoms of anemia*

Signs and Symptoms of anemia
Breathlessness
Chronic fatigue and weakness
Palpitations and tachycardia
Dizziness
Paleness
Loss of appetite
Depression
Irritability
Decreased muscle function
Impaired cognition
Loss of libido

Causes of anemia in patients with CKD

The causes of anemia in patients with CKD are various but clinically non-CKD related causes need to be ruled out. To diagnose anemia of CKD requires careful examination of the degree of anemia in relation to the degree of renal impairment. The evaluation of anemia in CKD patients should include, besides careful history taking and physical exam, a complete blood count with red blood cell indices (mean corpuscular Hb concentration (MCHC), mean corpuscular volume (MCV)), white blood cell count (including differential), reticulocytes and platelet count. Deficiency of iron, vitamin B12 or folates should be ruled out, especially in case of macrocytic anemia for the latter two causes. It is important to recognize other causes of anemia because it can reflect nutritional deficits, systemic illness or other conditions that require diagnosis and specific treatment. In this chapter, we focus on renal anemia, which is typically a normochromic, normocytic anemia without changes in leukocytes and platelets. The causes of renal anemia are summarized in table 2. Recently, several experimental in vivo and observational clinical studies suggest that vitamin D deficiency might be an additional co-factor of renal anemia. How vitamin D influences these different causes of anemia is discussed below.

Table 2. *Causes of renal anemia*

Causes of renal anemia	
1.	Iron deficiency
-	Abnormal iron absorption
-	Increased loss, especially in hemodialysis
-	Limited availability due to increased hepcidin concentrations
2.	EPO deficiency
3.	EPO resistance
4.	Abnormal HIF metabolism
5.	Hyperparathyroidism
6.	Anemia of chronic inflammation
7.	CKD related bone marrow suppression

Association between anemia and vitamin D

It is widely acknowledged that vitamin D plays an important role in bone and mineral metabolism. However, latest insights into the biological functions of vitamin D increased the interest in other clinical consequences of vitamin D deficiency. General population studies indicated a strong correlation between vitamin D deficiency and mortality and morbidity in patients with end-stage kidney failure treated with long-term hemodialysis (5;6). Moreover, vitamin D emerges as a potential important factor in erythropoiesis.

In hemodialysis population, vitamin D deficiency has been independently associated with erythropoietin hyporesponsiveness and anemia (7). In addition, several studies have shown that the administration of vitamin D or its analogues has been associated with an improvement of anemia and/or a decrease in erythropoietin (EPO) requirements. Also in patients with CKD not on dialysis, these associations are present (8). However, despite the clear epidemiological association between low vitamin D and anemia, the mechanism underlying this relationship has not been fully explained and several hypothesis are formulated how this link may be explained.

Iron deficiency and the role of vitamin D

The small polypeptide hepcidin is an important factor in the development of renal anemia. Hepcidin is the main regulatory protein of systemic iron metabolism and is mainly produced in the liver. It binds to ferroportin, a cellular iron exporter, which is located on the basolateral surface of gut enterocytes, the plasma membrane of reticuloendothelial cells (macrophages) and hepatocytes. Binding of hepcidin results in internalization and degradation of ferroportin limiting

the amount of iron release in the blood. The two major stimuli that are known to increase hepcidin levels are iron overload and (chronic) inflammation (fig 1). Since renal failure can be considered as a state of chronic inflammation, patients with CKD frequently have high serum levels of hepcidin resulting in so called 'functional' iron deficiency.

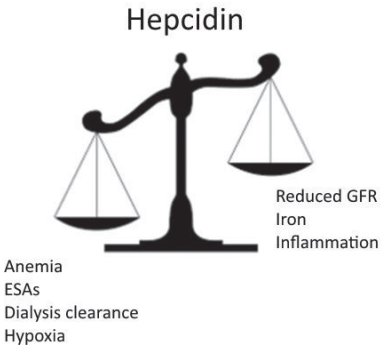


Figure 1. Different factors influencing the amount of hepcidin levels in blood. Conditions at the left suppress hepcidin, while those on the right increase it.

Recently, serum hepcidin concentrations were found to have an inverse association with serum vitamin D levels in CKD patients and a negatively association with hemoglobin and serum iron concentration (9;10). Given this link, several studies have been designed to explore the possible role for vitamin D in iron homeostasis. In vitro, Bacchetta et al. demonstrated that both in monocytes and hepatocytes, vitamin D is an important regulator of hepcidin expression (11). Treatment of cultured hepatocytes and monocytes with either prohormone 25-hydroxyvitamin D or active 1.25 dihydroxyvitamin D suppressed the expression of hepcidin and increased the expression of ferroportin. This in vitro effect was clinically studied by supplementing seven healthy volunteers with a single oral dose of vitamin D. Hepcidin levels decreased by 34% within the 24 hours following the vitamin D supplementation. The fact that vitamin D directly downregulates hepcidin expression can be explained on a molecular level by the presence of a vitamin D receptor (VDR) binding site on the promoter region of the human hepcidin gene, suggesting a gene suppressing effect. Further evidence for a role of vitamin D on hepcidin expression comes from a study done by Zughaier et al. (12). This in vitro experiment showed an association between vitamin D and decreased hepcidin expression in THP-1 (macrophage-like monocytic) cells in the presence of an inflammatory stimulus. Concurrently, vitamin D resulted in a dose dependent decrease in cytokines that increase hepcidin expression, like interleukin-6 (IL-6) and IL-1β. In vivo, vitamin D decreased systemic circulating hepcidin levels in humans with early stage CKD. Based on the current literature, one can conclude that high dose vitamin D therapy suppresses hepcidin expression directly, and indirectly by reducing hepcidin-inducing inflammatory cytokines IL-6 and IL-1β.

Chapter 5

Erythropoietin deficiency, resistance and the role of vitamin D

The red cell life span and the rate of red cell production are reduced in CKD and ideally the bone marrow compensates for this by increasing erythropoiesis. However, EPO-dependent compensatory mechanism is impaired due to failure to release the kidney-derived EPO in higher amounts leading to partial or complete erythropoietin deficiency. There are no endogenous stores of EPO.

Despite the treatment of renal anemia with iron and erythropoietin stimulating agents (ESA), many patients still remain anemic due to EPO hyporesponsiveness/resistance, defined as inability to meet the specified targets of Hb despite higher than usual doses of ESA's. The main causes for suboptimal response to ESA therapy are summarized in table 3.

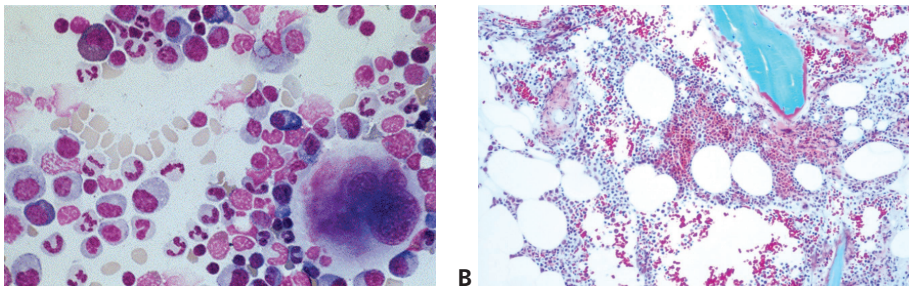
Causes for suboptimal response to ESA therapy
Iron deficiency (absolute and functional)
Infection/inflammation
Bleeding/hemolysis
Inadequate dialysis dose
Malignancy
Non-adherence with treatment therapies
Secondary hyperparathyroidism
Malnutrition
Bone marrow disorders/ hemoglobinopathies
Vitamin B12/ folate deficiency
Hypothyroidism
ACEi/ARB

Table 3. Causes for suboptimal responses to EPO therapy. Abbreviations are: ACEi, angiotensin converting enzyme inhibitor, ARB= angiotensin receptor blocker.

Five to 10% of EPO-treated patients exhibit an inadequate response to ESA's. It is well known that EPO hyporesponsiveness has an association with poor clinical outcomes, including cardiovascular morbidity, faster progression to end stage renal disease and all-cause mortality. Identification of factors that influence EPO responsiveness can optimize the management of anemia.

Erythropoiesis and vitamin D

Erythropoiesis is a complex process in the bone marrow resulting in the formation of mature red blood cells (RBC). This process is highly regulated so that, in non-disease states, the production of RBC's is equal to the destruction ensuring a constant red cell mass. Erythropoiesis is initiated when a pluripotent stem cell undergoes a series of subsequent differentiation steps in the hematopoietic environment. Stem cells and erythroid precursors are in intimate contact with stromal cells (adipocytes, fibroblasts, macrophages and endothelial cells), accessory cells (monocytes, T-lymphocytes) and the extracellular matrix. These stromal and accessory cells create a micro-environment in which the erythron cascade is regulated by growth factors and cytokines which have stimulatory or augmented effects on erythroid progenitors. This process can be negatively influenced under pathological conditions, such as inflammation, in which suppressive cytokines derives from accessory cells (tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and interleukin-6 (IL-6)) suppress the differentiation and proliferation (figure 2). Evidence for the effect of vitamin D on erythropoiesis comes from a study in which EPO was combined with or without vitamin D in cultured cells of patients with chronic uremia and in patients on chronic hemodialysis (13). In vitro, vitamin D increased the proliferation of erythroid precursors with a synergistic action when combined with EPO. This result was confirmed in 10 hemodialysis patients and seemed to be dose–dependent, synergistic with EPO but independent of parathyroid (PTH) suppression.



A B

Figure 2: histopathological morphology of (A) normal bone marrow with a normal erythropoietic cascade and (B) bone marrow of a CKD (stage 5D) patient with increased markers of inflammation, anemia and EPO resistance. An increase in stromal cells (adipocytes) is seen instead of hematopoietic cells. (Courtesy N. Bravenboer, VU university medical center).

Chapter 5

The role of Vitamin D on HIF metabolism

Hypoxia-inducible factor (HIF-1) is a heterodimeric transcription factor and regulate expression of genes in response to reduced oxygen tension, including genes required for erythropoiesis and iron metabolism. HIF consists of 2 subunits, HIF-1-α and HIF-1-β. At normal oxygen concentrations, the regulatory subunits are modified by iron-dependent prolyl hydroxylases (PHDs) resulting in rapid degradation of HIF-1-α. As oxygen tension decreases, the activities of PHDs are diminished and HIF-1-α accumulates and translocates into the nucleus, where it dimerizes with HIF-1-β. This leads to activation of transcriptional programs in response to hypoxia, resulting in increased production of erythropoietin and increased production of various proteins needed for effective iron transport, absorption and export from cells. These events, in conjunction with reduced serum hepcidin levels, enhance erythropoiesis.

The role of vitamin D in the HIF-1 pathway was described by Wong et al (14). While HIF-1-α is important in the activation of EPO expression, this study showed that vitamin D increased HIF-1-α gene expression through binding to its promoter in angiogenic myeloid cells of healthy volunteers. The effect of vitamin D on HIF-1-alpha in renal cells have never been tested, but clearly a vitamin D responsive element (VDRE) exist in the promoter region of the HIF gene. Since vitamin D activation and EPO production all occur in close proximity, conceivably a paracrine cross-talk between these systems may be at hand. Indeed, the recently published study concerning the effect of roxadustat, an orally bioavailable HIF-prolyl hydroxylases inhibitor (HIF-PH), confirmed the hypothesis that anemia in incident dialysis patients improves after administration of this protease inhibitor (15). Based on these studies, one could speculate that vitamin D supplementation can be used to partially restore renal anemia by suppression of HIF-PH , or increasing the expression of HIF-1-alpha. On the other hand, a study performed in various human cancer cells showed that vitamin D reduced the protein expression of HIF-1-α subunit and inhibited HIF-1 transcriptional activity (16). However, cancer cells are genetic abnormal and behave in a different way, which could explain these discrepant findings.

Besides involved in EPO expression, HIF-1-alpha might also couple iron sensing to iron regulation, as shown in an in vivo study in which iron deficient mice reveal an induction of HIF-1 and a decrease of hepcidin (17). The authors suggest that HIF-1-alpha may also bind to the hepcidin promoter as a gene suppressor, leading to decreased levels of hepcidin and consequently mobilizing iron to support erythrocyte production. HIF mobilizes iron to support erythrocyte production through a coordinated downregulation of hepcidin and upregulation of erythropoietin and ferroportin. A role for vitamin D in this mechanism has never been tested.

Vitamin D and inflammation

The majority of studies regarding vitamin D and renal anemia suggest a central role of inflammation in the mechanism underlying this association. Chronic low-grade inflammation is a hallmark of CKD, and produced inflammatory cytokines affect erythropoiesis. Vitamin D appears to modulate the level of systemic cytokine production resulting in attenuated severity of anemia of chronic disease. Detailed information about the relation between vitamin D and inflammation is beyond the scope of this chapter and we refer to chapter 21: Vitamin D and inflammation in CKD. In vivo and in vitro studies have demonstrated that calcitriol reduces cytokine production (18). Briefly summarized, vitamin D shows anti-inflammatory properties that improve responsiveness to EPO through the reduced production of hepcidin and pro-inflammatory cytokines. Immune cells express the vitamin D receptor (VDR) which, when activated, inhibits the expression of inflammatory cytokines like IL-1, IL-6, TNF-α and IFN-γ in accessory cells and in the serum. Additionally, VDR activation up-regulates the release of IL-10 from lymphocytes, which is an anti-inflammatory cytokine. It has been shown that calcitriol reduces cytokines production in human subjects as well (18) and in CKD patients vitamin D replacement could reduce this cytokine production leading to improved responsiveness to erythropoietin.

Vitamin D, VDR and the EPO receptor

Calcitriol exerts its functions by binding to the VDR, a member of receptors present in several tissues, that includes stromal and accessory cells in the bone marrow. The calcitriol-VDR complex binds transcriptional cofactors, which are able to interact with vitamin D responsive elements in gene promoter regions and regulate gene transcription. The uremic state in CKD affect the expression of VDR and VDR binding to vitamin D-responsive element in DNA (19). In addition, the VDR genotype also may influence all steps in the biological actions of calcitriol. Because calcitriol is involved in hematopoiesis, the question arises whether specific VDR receptor genotypes, might influence EPO responsiveness of CKD patients. Indeed, some VDR gene polymorphisms turned out to be protective against anemia and EPO hyporesponsiveness in hemodialysis patients (20;21). Erythropoietin receptor (EpoR) expression and activation are required for development of erythroid progenitor cells. The effect of 1,25(OH)2D3 and EPO on stem cell proliferation was studied by Alon et al (22). Calcitriol directly increased EpoR expression and synergistically stimulates cell proliferation along with EPO. There is evidence that vitamin D plays a role in EpoR expression, however the intracellular mechanisms by which 1,25(OH)2D3 upregulates the expression of EpoRs is not elucidated yet.

More evidence for a link between VDR and anemia comes from a study performed by Amato et al (23). This study evaluated the association between VDR polymorphisms and iron indices in 88 hemodialysis patients. They showed a significant association between a specific VDR allele and low transferrin saturation. Despite the fact that underlying mechanisms are not elucidated yet, these associations suggest a regulatory role of the vitamin D system in erythropoiesis and iron homeostasis.

Vitamin D and PTH

Since observational studies demonstrated a clear association between high serum levels of PTH and EPO resistance (24), the hypothesis emerged that hyperparathyroidism plays a role in the development of renal anemia. Since secondary hyperparathyroidism (SHPT) can in part be a consequence of vitamin D deficiency, PTH may indirectly contribute to the role of vitamin D deficiency in renal anemia. It is however somewhat controversial if excessive parathyroid activity per se causes anemia or alternatively is just a confounding feature of low levels of vitamin D, which then is the actual contributing factor to anemia. Four possible explanations have been proposed as to how SHPT might directly influence hemoglobin levels.

The most acknowledged effect of PTH on bone marrow cellularity is the induction of marrow fibrosis (osteitis fibrosa), which limits the space for red marrow and reduces the number of erythroid precursors. In a cross sectional study of 18 HD patients who had received EPO therapy for 1-3 years, bone histomorphometry was performed (25). The authors concluded that the dialysis patients with highest doses of EPO needed to achieve an adequate hematocrit response had significant higher serum PTH concentrations, higher percentages of osteoclastic and eroded bone surfaces and higher degree of bone marrow fibrosis. In contrast, Mandolfo et al showed that improvement of hemoglobin levels after parathyroidectomy (PTX) seems not to be related to improvement of marrow fibrosis but to the abrupt fall in PTH itself after surgery (26). Currently, discussion is still going on whether myelofibrosis is reversible after PTX and if so, at what time interval this can be expected. Because bone biopsy, necessary to diagnose bone marrow fibrosis, is an invasive method its use is generally restricted to a limited number of clinical indications in just a few dedicated clinical centers.

Another potential explanation for the relationship between SHPT and anemia could be the inhibitory effect of PTH on circulating EPO concentrations. Observations that plasma erythropoietin levels increase dramatically after parathyroidectomy point to a suppressive effect of PTH on the already reduced endogenous erythropoietin production in CKD (27). Washio et al. suggested the

role of both an abrupt fall in PTH and ionized calcium in the elevation of EPO, since partial PTX did not affect serum EPO levels (28). Currently, it is not clear whether PTH directly suppresses EPO production or the release of EPO in CKD.

The normal life span of a red blood cell (RBC) is approximately 100 days, but in CKD patients this life span is reduced. One of the causes could be the increased osmotic fragility of the RBC's in this patient group. RBC osmotic fragility is the diminished resistance to hemolysis due to osmotic changes and this is used to evaluate RBC friability. Wu et al found a significant relationship between increased serum PTH levels and RBC fragility in hemodialysis patients (Wu, 1998, red blood cell osmotic fragility in chronically hemodialyzed patients) . This could implicate that, in addition to dialysis therapy to improve uremic state, PTH reduction may improve the life span of the red blood cell and improve anemia.

Circulating EPO in the blood stream binds to EPO receptors on erythroblasts, which is necessary for normal RBC development. It is speculated that PTH has direct effect on this growth of RBC's, but evidence for the inhibitory effect of PTH on bone marrow erythropoiesis is sparse and contradictory. Better underpinned is the direct effect of vitamin D on erythropoiesis as discussed above.

Treatment of renal anemia

Treatment of renal anemia should be started based on individual patient symptoms and Hb concentrations. Since the development of recombinant human erythropoietin (epoetin alfa, EPO) and its derivatives in the 1980s followed by its approval by the US Food and Drug Administration (FDA), this has become the standard treatment of anemia employed in most patients with advanced CKD or end stage renal disease (ESRD). Initially it was assumed that near-normal levels of Hb would be advantageous. However, three landmark trials, i.e. CREATE (29) and CHOIR study (30) published in 2006 and the TREAT study (31) published in 2009, showed no superiority of full anemia correction by ESA. Conversely, these studies revealed an increased risk of progression to renal replacement therapy with a higher risk of mortality and cardiovascular morbidity and an increase in venous thromboembolic events. Secondary analyses of these trials showed that these risks might be especially present in patients with EPO hyporesponsiveness (32). Since iron depletion is one of the main causes of hyporesponsiveness to ESA as outlined above, the KDIGO guideline on 2012 recommends that iron therapy should be used to correct iron deficiency before initiating ESA therapy.

Currently, there is no international consensus regarding which route of administration of iron therapy is more appropriate to treat iron deficient anemia in CKD patients. To explore the optimal route of administration and dosing for iron therapy for the management of iron deficient anemia in patients with CKD not on dialyses, with or without concomitant ESA therapy, the FIND-CKD study was performed (33). This multicenter, prospective and randomized study was performed among 626 patients who received intravenous ferric carboxymaltose (FCM) targeting a higher (400-600 µg/L) or lower (100-200 µg/L) ferritin or oral iron therapy. The authors concluded that, compared with oral iron, IV FCM targeting a ferritin of 400-600 µg/L was superior to oral iron in delaying and/or reducing the need for other anemia management including ESA during this 12 month study. This study was not powered to assess safety end points, however, high ferritin FCM was well tolerated with no important adverse events.

Several small studies show that the administration of vitamin D or its analogues are associated with an improvement of anemia or a reduction in EPO requirements. Calcitriol improved Hb levels and reduced the need for EPO in CKD patients and HD patients (13;34), while alfacalcidol (35), cholecalciferol and ergocalciferol induced higher levels of Hb in hemodialysis patients (36). However, large and prospective randomized trials aiming to improve anemia in CKD as primary endpoint, using any form of vitamin D are still lacking. The largest randomized trial in this field was performed in 60 CKD patients stage 3B-5 and anemia to determine whether paricalcitol, compared to calcitriol, improved anemia (37). These patients, with normal PTH levels and without signs of clinical inflammation, were randomized in two groups to receive low doses calcitriol or paricalcitol for 6 months. During this period, paricalcitol resulted in a significant increase in Hb concentration, without a change in iron balance, inflammatory markers and PTH plasma concentration. However, patients treated with calcitriol showed a decrease in Hb levels. Due to the lack of a control group in this study, it is impossible to draw conclusions about the role of vitamin D in the overall management of anemia in patients with CKD.

In conclusion, epidemiological data and biological mechanisms suggest that active vitamin D could have a positive effect on renal anemia. Currently however, the clinical relevance of this is unsure. In our opinion, it is too early to conclude that active vitamin D administration improves renal anemia in CKD patients. It is conceivable though, that it may be considered in patients with unexplained EPO-hyporesponsiveness.

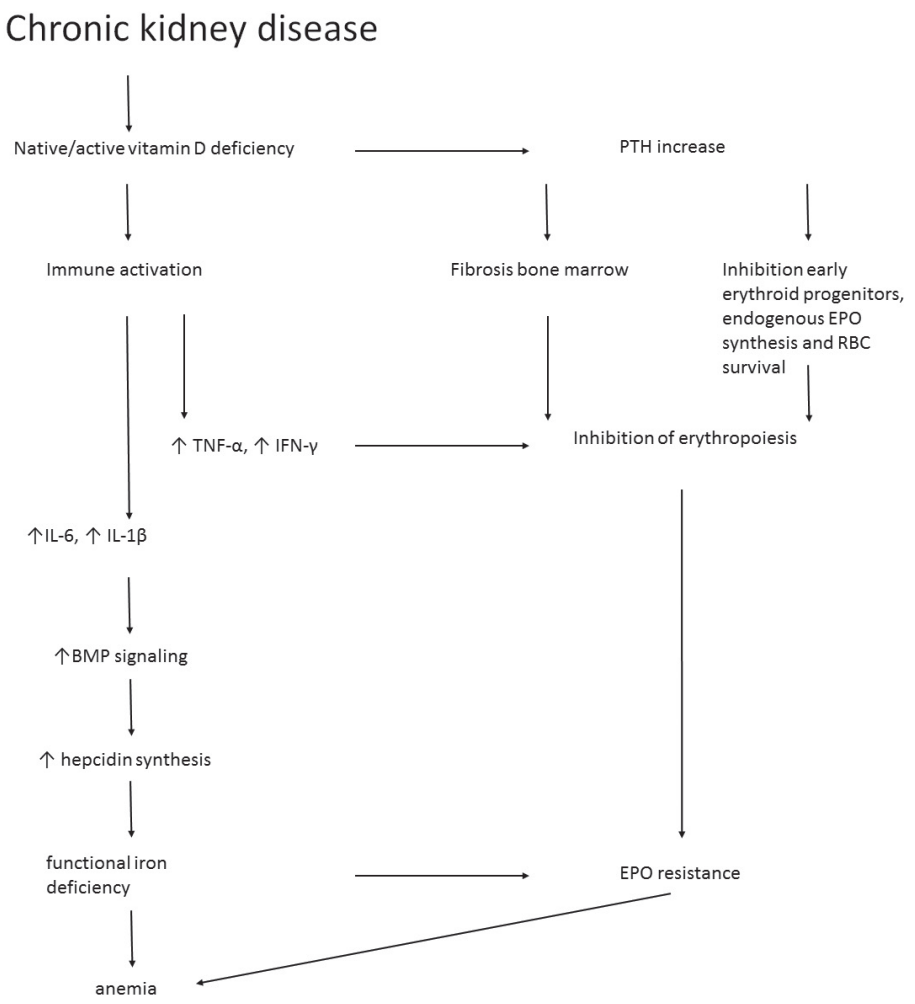


Figure 3. Effects of Vitamin D deficiency in CKD on the immune system and EPO-resistant anemia.

References

1. McClellan W, Aronoff SL, Bolton WK, Hood S, Lorber DL, Tang KL, et al. The prevalence of anemia in patients with chronic kidney disease. Curr Med Res Opin 2004 Sep;20(9):1501-10.

2. Levin A, Thompson CR, Ethier J, Carlisle EJ, Tobe S, Mendelssohn D, et al. Left ventricular mass index increase in early renal disease: impact of decline in hemoglobin. Am J Kidney Dis 1999 Jul;34(1):125-34.

3. Sarnak MJ, Tighiouart H, Manjunath G, Macleod B, Griffith J, Salem D, et al. Anemia as a risk factor for cardiovascular disease in The Atherosclerosis Risk in Communities (ARIC) study. J Am Coll Cardiol 2002 Jul 3;40(1):27-33.

4. McClellan WM, Flanders WD, Langston RD, Jurkovitz C, Presley R. Anemia and renal insufficiency are independent risk factors for death among patients with congestive heart failure admitted to community hospitals: a population-based study. J Am Soc Nephrol 2002 Jul;13(7):1928-36.

5. Wolf M, Shah A, Gutierrez O, Ankers E, Monroy M, Tamez H, et al. Vitamin D levels and early mortality among incident hemodialysis patients. Kidney Int 2007 Oct;72(8):1004-13.

6. Matias PJ, Ferreira C, Jorge C, Borges M, Aires I, Amaral T, et al. 25-Hydroxyvitamin D3, arterial calcifications and cardiovascular risk markers in haemodialysis patients. Nephrol Dial Transplant 2009 Feb;24(2):611-8.

7. Kiss Z, Ambrus C, Almasi C, Berta K, Deak G, Horonyi P, et al. Serum 25(OH)-cholecalciferol concentration is associated with hemoglobin level and erythropoietin resistance in patients on maintenance hemodialysis. Nephron Clin Pract 2011;117(4):c373-c378.

8. Patel NM, Gutierrez OM, Andress DL, Coyne DW, Levin A, Wolf M. Vitamin D deficiency and anemia in early chronic kidney disease. Kidney Int 2010 Apr;77(8):715-20.

9. Carvalho C, Isakova T, Collerone G, Olbina G, Wolf M, Westerman M, et al. Hepcidin and disordered mineral metabolism in chronic kidney disease. Clin Nephrol 2011 Aug;76(2):90-8.

10. Perlstein TS, Pande R, Berliner N, Vanasse GJ. Prevalence of 25-hydroxyvitamin D deficiency in subgroups of elderly persons with anemia: association with anemia of inflammation. Blood 2011 Mar 10;117(10):2800-6.

11. Bacchetta J, Zaritsky JJ, Sea JL, Chun RF, Lisse TS, Zavala K, et al. Suppression of iron-regulatory hepcidin by vitamin D. J Am Soc Nephrol 2014 Mar;25(3):564-72.

12. Zughaier SM, Alvarez JA, Sloan JH, Konrad RJ, Tangpricha V. The role of vitamin D in regulating the iron-hepcidin-ferroportin axis in monocytes. J Clin Transl Endocrinol 2014 Mar 21;1(1):19-25.

13. Aucella F, Scalzulli RP, Gatta G, Vigilante M, Carella AM, Stallone C. Calcitriol increases burst-forming unit-erythroid proliferation in chronic renal failure. A synergistic effect with r-HuEpo. Nephron Clin Pract 2003;95(4):c121-c127.

14. Wong MS, Leisegang MS, Kruse C, Vogel J, Schurmann C, Dehne N, et al. Vitamin D promotes vascular regeneration. Circulation 2014 Sep 16;130(12):976-86.

15. Besarab A, Chernyavskaya E, Motylev I, Shutov E, Kumbar LM, Gurevich K, et al. Roxadustat (FG-4592): Correction of Anemia in Incident Dialysis Patients. J Am Soc Nephrol 2015 Oct 22.

16. Ben-Shoshan M, Amir S, Dang DT, Dang LH, Weisman Y, Mabjeesh NJ. 1alpha,25-dihydroxyvitamin D3 (Calcitriol) inhibits hypoxia-inducible factor-1/vascular endothelial growth factor pathway in human cancer cells. Mol Cancer Ther 2007 Apr;6(4):1433-9.

17. Peyssonnaux C, Zinkernagel AS, Schuepbach RA, Rankin E, Vaulont S, Haase VH, et al. Regulation of iron homeostasis by the hypoxia-inducible transcription factors (HIFs). J Clin Invest 2007 Jul;117(7):1926-32.

18. Blazsek I, Farabos C, Quittet P, Labat ML, Bringuier AF, Triana BK, et al. Bone marrow stromal cell defects and 1 alpha,25-dihydroxyvitamin D3 deficiency underlying human myeloid leukemias. Cancer Detect Prev 1996;20(1):31-42.

19. Glorieux G, Vanholder R. Blunted response to vitamin D in uremia. Kidney Int Suppl 2001 Feb;78:S182-S185.

20. Sezer S, Tutal E, Bilgic A, Ozdemir FN, Haberal M. Possible influence of vitamin D receptor gene polymorphisms on recombinant human erythropoietin requirements in dialysis patients. Transplant Proc 2007 Jan;39(1):40-4.

21. Erturk S, Kutlay S, Karabulut HG, Keven K, Nergizoglu G, Ates K, et al. The impact of vitamin D receptor genotype on the management of anemia in hemodialysis patients. Am J Kidney Dis 2002 Oct;40(4):816-23.

22. Alon DB, Chaimovitz C, Dvilansky A, Lugassy G, Douvdevani A, Shany S, et al. Novel role of 1,25(OH)(2)D(3) in induction of erythroid progenitor cell proliferation. Exp Hematol 2002 May;30(5):403-9.

23. Amato M, Pacini S, Aterini S, Punzi T, Gulisano M, Ruggiero M. Iron indices and vitamin D receptor polymorphisms in hemodialysis patients. Adv Chronic Kidney Dis 2008 Apr;15(2):186-90.

24. Falko JM, Guy JT, Smith RE, Mazzaferri EL. Primary hyperparathyroidism and anemia. Arch Intern Med 1976 Aug;136(8):887-9.

25. Rao DS, Shih MS, Mohini R. Effect of serum parathyroid hormone and bone marrow fibrosis on the response to erythropoietin in uremia. N Engl J Med 1993 Jan 21;328(3):171-5.

26. Mandolfo S, Malberti F, Farina M, Villa G, Scanziani R, Surian M, et al. Parathyroidectomy and response to erythropoietin therapy in anaemic patients with chronic renal failure. Nephrol Dial Transplant 1998 Oct;13(10):2708-9.

27. Urena P, Eckardt KU, Sarfati E, Zingraff J, Zins B, Roullet JB, et al. Serum erythropoietin and erythropoiesis in primary and secondary hyperparathyroidism: effect of parathyroidectomy. Nephron 1991;59(3):384-93.

28. Washio M, Iseki K, Onoyama K, Oh Y, Nakamoto M, Fujimi S, et al. Elevation of serum erythropoietin after subtotal parathyroidectomy in chronic haemodialysis patients. Nephrol Dial Transplant 1992;7(2):121-4.

29. Drueke TB, Locatelli F, Clyne N, Eckardt KU, Macdougall IC, Tsakiris D, et al. Normalization of hemoglobin level in patients with chronic kidney disease and anemia. N Engl J Med 2006 Nov 16;355(20):2071-84.

30. Singh AK, Szczech L, Tang KL, Barnhart H, Sapp S, Wolfson M, et al. Correction of anemia with epoetin alfa in chronic kidney disease. N Engl J Med 2006 Nov 16;355(20):2085-98.

31. Pfeffer MA, Burdmann EA, Chen CY, Cooper ME, de ZD, Eckardt KU, et al. A trial of darbepoetin alfa in type 2 diabetes and chronic kidney disease. N Engl J Med 2009 Nov 19;361(21):2019-32.

32. Kilpatrick RD, Critchlow CW, Fishbane S, Besarab A, Stehman-Breen C, Krishnan M, et al. Greater epoetin alfa responsiveness is associated with improved survival in hemodialysis patients. Clin J Am Soc Nephrol 2008 Jul;3(4):1077-83.

33. Macdougall IC, Bock AH, Carrera F, Eckardt KU, Gaillard C, Van WD, et al. FIND-CKD: a randomized trial of intravenous ferric carboxymaltose versus oral iron in patients with chronic kidney disease and iron deficiency anaemia. Nephrol Dial Transplant 2014 Nov;29(11):2075-84.

34. Goicoechea M, Vazquez MI, Ruiz MA, Gomez-Campdera F, Perez-Garcia R, Valderrabano F. Intravenous calcitriol improves anaemia and reduces the need for erythropoietin in haemodialysis patients. Nephron 1998;78(1):23-7.

35. Albitar S, Genin R, Fen-Chong M, Serveaux MO, Schohn D, Chuet C. High-dose alfacalcidol improves anaemia in patients on haemodialysis. Nephrol Dial Transplant 1997 Mar;12(3):514-8.

36. Saab G, Young DO, Gincherman Y, Giles K, Norwood K, Coyne DW. Prevalence of vitamin D deficiency and the safety and effectiveness of monthly ergocalciferol in hemodialysis patients. Nephron Clin Pract 2007;105(3):c132-c138.

37. Riccio E, Sabbatini M, Bruzzese D, Capuano I, Migliaccio S, Andreucci M, et al. Effect of paricalcitol vs calcitriol on hemoglobin levels in chronic kidney disease patients: a randomized trial. PLoS One 2015;10(3):e0118174.

Chapter 6

VITAMIN D RECEPTOR ACTIVATOR AND DIETARY SODIUM RESTRICTION TO REDUCE RESIDUAL URINARY ALBUMIN EXCRETION IN CHRONIC KIDNEY DISEASE (VIRTUE STUDY): RATIONALE AND STUDY PROTOCOL

Charlotte A. Keyzer[1], Maarten A. de Jong[1], G. Fenna van Breda[2], Marc G. Vervloet[2], Gozewijn D. Laverman[3], Marc Hemmelder[4], Wilbert M. Janssen[5], Hiddo J. Lambers Heerspink[6], Gerjan Navis[1] and Martin H. de Borst[1] for the Holland Nephrology Study (HONEST) network

1. Department of Internal Medicine, Division of Nephrology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands;
2. Department of Nephrology and Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, the Netherlands;
3. Department of Internal Medicine, Division of Nephrology, ZGT Hospital, Almelo, the Netherlands;
4. Department of Internal Medicine, Division of Nephrology, Medical Center Leeuwarden, Leeuwarden, the Netherlands;
5. Department of Internal Medicine, Division of Nephrology, Martini Hospital Groningen, Groningen, the Netherlands;
6. Department of Clinical Pharmacy and Pharmacology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands

Nephrol Dial Transplant 2015; Mar 16; doi: 10.1093/ndt/gfv033

Abstract

Background. Optimal albuminuria reduction is considered essential to halt chronic kidney disease (CKD) progression. Both vitamin D receptor activator (VDRA) treatment and dietary sodium restriction potentiate the efficacy of renin-angiotensin-aldosterone-system (RAAS)-blockade to reduce albuminuria. The ViRTUE study addresses whether a VDRA in combination with dietary sodium restriction provides further albuminuria reduction in non-diabetic CKD patients on top of RAAS-blockade.

Methods/design. The ViRTUE study is an investigator-initiated, prospective, multi-centre, randomized, double-blind (paricalcitol versus placebo), placebo-controlled trial targeting stage 1-3 CKD patients with residual albuminuria of >300 mg/day due to non-diabetic glomerular disease, despite angiotensin converting enzyme inhibitor or angiotensin receptor blocker use. During run-in, all subjects switched to standardized RAAS-blockade (ramipril 10 mg/day) and blood pressure titrated to <140/90 mmHg according to a standardized protocol. Eligible patients are subsequently enrolled and undergo four consecutive study periods in random order of 8 weeks each: (i) paricalcitol (2 µg/day) combined with a liberal sodium diet (~200 mmol Na^+/day, i.e. mean sodium intake in the general population), (ii) paricalcitol (2 µg/day) combined with dietary sodium restriction (target: 50 mmol Na^+/day), (iii) placebo combined with a liberal sodium diet, and (iv) placebo combined with dietary sodium restriction. Data are collected at the end of each study period. The primary outcome is 24-h urinary albumin excretion. Secondary study outcomes are blood pressure, renal function (estimated glomerular filtration rate), plasma renin activity and, in a sub-population (N=9), renal haemodynamics (measured glomerular filtration rate and effective renal plasma flow). A sample size of 50 patients provides 90% power to detect 23% reduction in albuminuria, assuming a 25% dropout rate.

Discussion. Further reduction of residual albuminuria by combination of VDRA treatment and sodium restriction during single-agent RAAS-blockade will justify long-term studies on cardiorenal outcomes and safety.

Clinical trial registration. NTR2898 (Dutch trial register).

Rationale

Albuminuria independently contributes to chronic kidney disease (CKD) progression towards end-stage renal disease (ESRD)[1], and is also an independent predictor of cardiovascular outcome[2]. Optimal lowering of albuminuria is therefore a cornerstone of current CKD treatment. Pharmacological blockade of the renin-angiotensin-aldosterone system (RAAS) by angiotensin converting enzyme (ACE) inhibition or angiotensin receptor blockade (ARB) reduces albuminuria and blood pressure, retards renal disease progression and reduces cardiovascular risk in CKD patients.[3,4] Despite optimally dosed single agent RAAS-blockade, however, considerable residual albuminuria remains in many CKD patients. The amount of residual albuminuria is closely related to long-term renal and cardiovascular prognosis.[5,6] Consequently, further albuminuria reduction by means of adjunct pharmacological or dietary measures has been advocated to further improve cardiorenal outcomes. Recent studies have shown that dual RAAS-blockade using traditional RAAS-inhibitors did not result in increased renoprotection compared with single-agent RAAS-blockade.[7-9] Rather, particularly in diabetic nephropathy patients, dual RAAS blockade was accompanied by an increased risk of acute kidney injury and hyperkalemia.[8-10] Because dual RAAS-blockade is now considered insufficiently safe for a considerable part of the CKD population, it is necessary to find alternative treatment modalities with a more attractive efficacy/side effect ratio.

Dietary sodium restriction potentiates the antiproteinuric efficacy of RAAS-blockade. A modest reduction of dietary sodium intake to 2 g/day is associated with a 30% proteinuria reduction, which is in the same order of magnitude as the response to single RAAS-blockade.[11] Combining RAAS-blockade with sodium restriction synergistically reduces proteinuria in non-diabetic CKD patients.[11,12] Sodium restriction on top of RAAS-blockade is also associated with long-term renal and cardiovascular protective effects both in non-diabetes and diabetes.[13,14] Conversely, sodium overload may even annihilate the antihypertensive and antiproteinuric effects of RAAS-blockade.[15] Despite adequate sodium restriction during single-agent RAAS-blockade, however, residual proteinuria may still remain, requiring additional intervention.

Recent preclinical[16] and clinical[17] studies demonstrated that vitamin D receptor activators (VDRA, e.g. paricalcitol) may provide additional renoprotection by reducing residual albuminuria. The renoprotective effects of VDRA may at least partly be mediated by the RAAS.[18-20] Vitamin D receptor activation directly suppresses renin gene transcription.[21] Additionally active vitamin D has displayed anti-inflammatory and antifibrotic effects as well as specific beneficial effects on podocytes in models of CKD (reviewed by *Mirkovic et al*[16] and *Ito et al*[22]). Thus, the renoprotective effects of VDRA seem to be set forth partly beyond the RAAS, and because VDRAs are not

accompanied by (major) effects on blood pressure[23] or serum potassium, these agents are attractive adjuncts to RAAS-blockade. Indeed, renoprotective effects of VDRA treatment appear additive to RAAS-blockade effects, both in the clinical setting and in animal studies.[16,17]

Whether the capacity of VDRA treatment to lower residual albuminuria depends on sodium intake is unclear. Surprisingly, a post-hoc analysis of the VITAL study suggested that patients with higher baseline dietary sodium intake displayed a stronger antiproteinuric effect upon VDRA treatment.[24] This would be in contrast with a large number of reports demonstrating that sodium restriction potentiates the antiproteinuric efficacy of RAAS-blockade, but also other classes of drugs such as non-steroidal anti-inflammatory drugs[25] and vasopeptidase inhibitors[26]. Moreover, we recently demonstrated that dietary sodium restriction indeed potentiates the antiproteinuric effect of the VDRA paricalcitol in a rat model of proteinuric nephropathy[27].

To prospectively study the potentially interacting effects of dietary sodium intake and VDRA treatment on residual albuminuria during background RAAS-blockade in patients, we designed a double-blind randomized placebo-controlled crossover trial with a 2x2 factorial design. The trial consists of four study periods comparing residual albuminuria during treatment with the VDRA paricalcitol or placebo during a low or liberal sodium diet, respectively, all during background ACE inhibition in CKD patients with residual albuminuria due to non-diabetic glomerular disease. Diabetic CKD patients are not included because vitamin D may interfere with insulin secretion and insulin sensitivity (reviewed by *De Boer et al*[28]) which could also influence residual albuminuria, and thus cause heterogeneity in the results. Furthermore, the ViRTUE focuses on patients with albuminuria from glomerular origin. Therefore, patients with secondary albuminuria due to disease such as amyloidosis, multiple myeloma or cancer are also excluded from participation in this trial. In a substudy, we will investigate the effect of paricalcitol and dietary sodium restriction on renal haemodynamics, *i.e.* measured glomerular filtration rate (GFR) and effective renal plasma flow (ERPF), given the previously documented effect of paricalcitol on estimated glomerular filtration rate (eGFR)[17]. Successful reduction of residual albuminuria by VDRA treatment in combination with dietary sodium restriction during single RAAS-blockade may pave the way for a large-scale clinical trial providing evidence for long-term beneficial effects of this combination therapy on cardiorenal endpoints.

Study protocol

Study design and organization

The ViRTUE study is an investigator-initiated, prospective, multi-centre, randomized, double-blind, placebo-controlled trial targeting non-diabetic CKD patients with residual albuminuria despite single-agent RAAS-blockade. The ViRTUE study is conducted according to the principles of the Declaration of Helsinki and in accordance with the Medical Research Involving Human Subjects Act (WMO, The Netherlands). The study has been approved by the Medical Ethical Committee of the University Medical Center Groningen, the Netherlands (METc 2009.272), and has been registered in the Dutch clinical trial register (NTR2898). Participation in the study is on voluntary basis. Patients will not receive any financial support or priority for treatment of other diseases during this study.

Participants

The ViRTUE study recruited stage 1-3 non-diabetic CKD patients with residual albuminuria >300 mg/day due to (non-diabetic) glomerular disease despite optimally dosed single-agent RAAS-blockade. Recruitment took place at five academic or non-academic hospitals in the Netherlands. Patients were required to have a stable renal function with a creatinine clearance >30 mL/min, the average of two parathyroid hormone (PTH) values should be <1.5 times the upper limit of normal (defined by the reference values of each participating centre) and the average of two corrected serum calcium values should be between 2.0 and 2.6 mmol/L. Furthermore, patients should not have received (within 3 months prior to screening) vitamin D (analogues). Circulating 25(OH)D was not used as specific criteria, because previous studies suggest that the albuminuria-lowering effect of vitamin D receptor activation is independent of the vitamin D status as reflected by 25(OH)D levels (VITAL study, unpublished data). This approach is similar to the previous clinical trials with paricalcitol among CKD patients.[23,24,29] Patients who also met the other prespecified eligibility criteria (Table 1) and provided written informed consent were enroled. Patient enrolment started March 2012 and was closed in May 2014.

Table 1. Eligibility criteria of the ViRTUE study

	Inclusion criteria		Exclusion criteria
1	Male and female patients	**1**	Diabetes Mellitus
2	Non-diabetic glomerular disease as established by history, serum biochemistry tests and/or renal biopsy	**2**	Uncontrolled hypertension
3	Age ≥18 years	**3**	Hyperkalemia (potassium >6.0 mmol/L)
4	Residual albuminuria >300 mg/day and <10 g/day during conventional treatment of at least 8 weeks with ACEi or ARB at the maximum recommended dose	**4**	Cardiovascular disease (myocardial infarction, unstable angina, percutanous transluminal coronary angioplasty, coronary artery bypass grafting, or stroke within last 6 months, heart failure NYHA III-IV)
5	Stable renal function (creatinine clearance of >30 ml/min; with <6 mL/min per year decline)	**5**	Epilepsy
6	Average of 2 consecutive PTH values of < 1.5 times the upper limit of normal (defined by the reference values of each participating center), 2 consecutive serum calcium levels between 2.0 and 2.6 mmol/L (corrected for albumin levels), 2 consecutive serum phosphorus levels of ≤ 1.5 mmol/L within 4 weeks prior to treatment	**6**	Liver disease resulting in aberrations of liver function tests
7	Self-written informed consent (no incapacitated adults)	**7**	Previously treated (within 3 months of screening) with paricalcitol or vitamin D (analogue)
		8	Contraindication to ACEi, high/low-sodium diet or paricalcitol
		9	Medication interacting with ACEi or paricalcitol
		10	Frequent NSAID use (> 2 doses/week), use of immunosuppressive drugs or use of digoxin
		11	Active malignancy
		12	Any bowel disorder resulting in fat malabsorption
		13	Pregnant or nursing (lactating) women, where pregnancy is defined as a state of a female after conception and until the termination of gestation, confirmed by a positive ß-hCG laboratory test (>5 mIU/ml)
		14	Incompliance with diet or study medication
		15	Any psychiatric condition or psychiatric medication use
		16	Drug or alcohol abuse

Abbreviations: ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; ß-hCG, beta human chorionic gonadotropin; NSAID, non-steroidal anti-inflammatory drug; NYHA, New York Heart Association; PTH, parathyroid hormone.

Run-in period

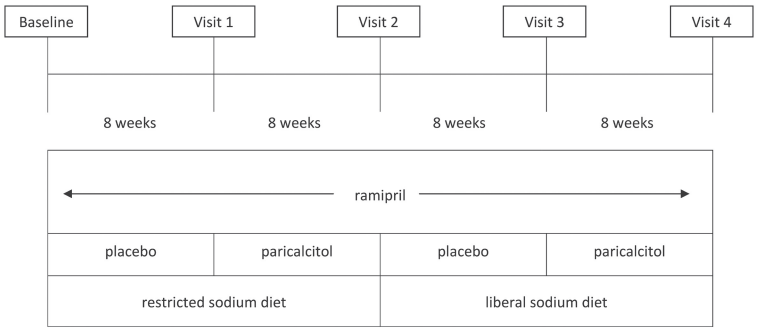
Before study entry, patients start with a wash-out/wash-in period in which RAAS-blocking agents and diuretics (except for furosemide) are discontinued, and standardized RAAS-blockade (10 mg ramipril/day) is started to titrate blood pressure to a value of <140/90 mmHg. If necessary, pharmacological antihypertensive therapy is optimized during the run-in period. Blood pressure is evaluated during every outpatient clinic visit under constant conditions, at 1-min intervals for 15 min by an automatic device (Dinamap; DE Medical systems, Milwaukee, WI), with the patient in a semi-supine position. If the target blood pressure of <140/90 mmHg is not reached within 6 weeks after the initiation of ramipril, additional antihypertensive medication (metoprolol, doxazosin and/or amlodipine) is added to the treatment regimen with 4-week intervals. Blood pressure is evaluated every fourth week, and patients with adequate blood pressure values enrol in the study. After a maximum wash-in/wash-out period of 18 weeks, patients with a blood

pressure value < 180/100 mmHg will be able to enrol in the study. Patients with a blood pressure value of >180/100 mmHg, despite optimal antihypertensive treatment as indicated above, are not included in the study.

Study period

Patients are subjected to four consecutive study periods in random order with duration of 8 weeks each. These study periods (Figure 1) consist of: (i) the VDRA paricalcitol (19-nor-1,25[OH]$_2$-vitamin D2, 2 µg/day[24,30]) combined with a liberal sodium diet (~200 mmol Na+/d [~4.8 g], *i.e.* corresponding to the mean sodium intake in the general population), (ii) paricalcitol (2 µg/day) combined with dietary sodium restriction (target 50 mmol Na^+/day, ~1.2 g), (iii) placebo combined with a liberal sodium diet, and (iv) placebo combined with dietary sodium restriction. The duration of each treatment period is based on previous studies with paricalcitol demonstrating maximum albuminuria reduction at 4-6 weeks after treatment initiation.[24,31] Since our 8-week study periods are considerably longer than the wash-out of the interventions (paricalcitol < 2 weeks[31] and re-establishment of steady state after a change in sodium diet < 2 weeks[11,12]), the protocol does not include wash-out periods.

Patients are instructed to take the study medication once daily, in the morning, except for study days, when the study drug will be taken after data have been collected at the study centre. Every 8 weeks, patients collect 24-h urine, and after an overnight fast blood pressure is measured and blood and spot urine samples are taken (see Figure 1). Collected data at the end of each 8-week treatment period are used for analysis. At 4 weeks from start of the treatment period, serum albumin, calcium, phosphorus and PTH are measured for safety analyses.



	Baseline	Week 4	Week 8	Week 12	Week 16	Week 20	Week 24	Week 28	Week 32
Monitor adverse events			X		X		X		X
Monitor vital signs	X		X		X		X		X
Complete chemistry	X		X		X		X		X
Limited chemistry*		X		X		X		X	
24h urine collection	X	X	X	X	X	X	X	X	X

* serum albumin, calcium, phosphate and parathyroid hormone.

Figure 1. Study design of the ViRTUE study. Patients are subjected to four consecutive study periods in random order with duration of 8 weeks each. The interventions are paricalcitol (2 µg/day) or placebo combined with a liberal sodium diet or dietary sodium restriction.

During the course of the study, patients will receive a thorough monitoring of compliance to the sodium diet by measuring 24-h urinary sodium excretion every 4 weeks. Patients will be motivated to ensure a stable protein intake (1.1 g/kg body weight per day) during the periods of different sodium intake. Between inclusion and start of the study, patients will be asked to keep a dietary diary for a period of 3 days and to collect 24-h urine on the third day. The results of the dietary diary and the 24-h urine collection will be used during a dietary consult in which the patient will receive personal dietary advice to be compliant to the sodium-restricted diet. Differences in sodium intake between the study periods will be achieved by replacing sodium-rich products with a low-sodium product of the same product group to maintain isocaloric intake with a similar balance among protein, carbohydrate and fat. When subjects report symptomatic hypotension during the study period, especially while on dietary sodium restriction, the dose or the number of antihypertensives will be reduced. If blood pressure afterwards rises to >140/90 mmHg, the dose or number of antihypertensives will be restored.

Renal haemodynamics substudy

In a substudy consisting of male patients, we will evaluate the effect of paricalcitol and dietary sodium restriction on renal haemodynamics (GFR and ERPF). All male patients participating at the University Medical Center Groningen or Martini Hospital Groningen were asked informed consent for this sub-study. At the end of each study period, subjects undergo GFR/ERPF measurements remaining in a semi-supine position except during voiding as previously described in more detail[32].

In short, to ensure sufficient urine output, subjects will be provided with 250 ml of oral fluids every hour. After a 1.5-h stabilization period, GFR and ERPF are measured as the clearances of constantly infused ^{125}I-iothalamate and ^{131}I-hippuran, respectively. After the stabilization period, blood is drawn every hour and urine is collected every two hours. In this set-up, GFR is measured as the urinary clearance of ^{125}I-iothalamate using the formula $(U \times V)/P$ (where U is concentration per mL urine, V is urinary flow rate in ml/min and P is plasma concentration) and corrected for voiding errors by the ratio of plasma to urinary clearance of ^{131}I-hippuran. Furthermore, 24-h ambulatory blood pressure is measured in the week prior to the GFR/ERPF measurement using a Spacelabs 90217 (Spacelabs Medical Products, Sydney, Australia) device with blood pressure and heart rate recorded three times per hour throughout awake periods and once every hour during sleeping periods.

Randomization and blinding

To prevent systematic errors resulting from the crossover design, the different periods, treatment (placebo or paricalcitol) as well as diet (low or liberal sodium diet), will be randomized for each patient. We defined four different treatment sequences (see Figure 2). Randomization of these sequences was performed externally by the pharmaceutical company that delivered the study medication (AbbVie).

Administration of study medication (placebo or paricalcitol) takes place in a double-blinded fashion, while the diet (low or liberal sodium) will be open label. Unblinding is only acceptable when severe deterioration of renal function (defined as ≥25% renal function decline between two visits) is recorded or when a serious adverse event occurs that requires information regarding the study medication use (paricalcitol or placebo).

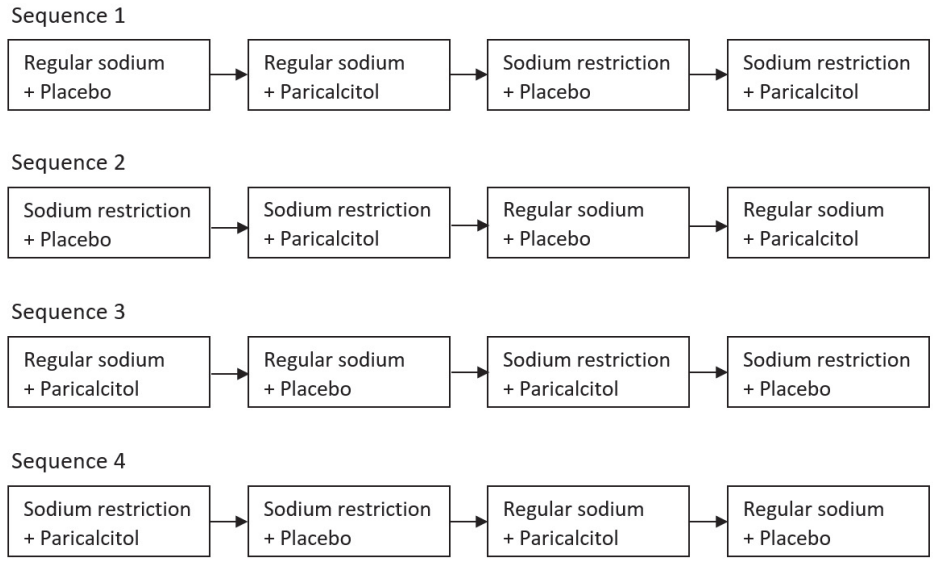


Figure 2. Four different sequences of treatment periods in the ViRTUE study. The interventions are a liberal sodium diet or dietary sodium restriction in combination with paricalcitol (2 µg/day) or placebo.

Safety

In general, the risk for participation in this study is estimated to be low. Paricalcitol has been approved by the European Medicines Agency (EMA), the U.S. Food and Drug Administration (FDA) and the Dutch Medicines Evaluation Board (MEB) and is widely prescribed in the clinical setting, mainly for the treatment of secondary hyperparathyroidism and renal osteodystrophy in patients with advanced CKD. No serious side effects are expected at the paricalcitol dosage used in this study, expect for hypercalcaemia. Possible other side effects of paricalcitol include stomach complaints, skin rash, dizziness, taste abnormalities, constipation, dry mouth, itching, urticaria, muscle spasms, intolerance or liver abnormalities; these side effect are rare and mild. Four weeks after the start of a treatment period, serum albumin, calcium and PTH are measured for a safety analysis. In case of hypercalcaemia (corrected serum calcium >2.60 mmol/l) or hypoparathyroidism (PTH <1.5 pmol/L), the dose of the study medication (paricalcitol or placebo) is reduced from two capsules to one capsule per day for the remaining study period(s). If hypercalcaemia or hypoparathyroidism as defined above persists, the patient is withdrawn for the study. Study medication is also discontinued if the investigator determines that continuing the drug would result in a significant safety risk for the patient or if the study drug would be considered detrimental to the patient's well-being.

Use of any (other) RAAS-blocking agent, diuretics (except for furosemide), ketoconazole and antacids is not allowed after the start of the study as these medications may interfere with the

evaluation of safety, tolerability and/or efficacy of the study medication. Furosemide is tolerated during the study since comorbidity (e.g. oedema) may require diuretic therapy to be continued throughout the study.

All patient-reported or observed adverse events are recorded. There are no predefined criteria for premature termination of the study, excepted for hypercalcaemia or hypoparathyroidism as defined above. If, however, during the conductance of the study new information becomes available showing that continuation of the study would result in a significant safety risk for the patients, the principal investigator and project leader will decide to terminate the study.

Study endpoints

The primary study endpoint is albuminuria, measured in a 24-h urine portion collected at the end of each study period. Secondary study endpoints are blood pressure (systolic, diastolic and mean arterial pressure), renal function (creatinine clearance and eGFR by creatinine-based CKD-EPI formula), urinary sodium excretion (assessment of dietary sodium intake), plasma renin activity and in the substudy renal haemodynamics (measured GFR and ERPF). Other prespecified exploratory parameters may include body mass index, circulating levels of calcium, phosphate, sodium, potassium, urea, cholesterol, triglycerides, total protein and albumin, aldosterone, 25(OH) and 1,25$(OH)_2$ vitamin D, vitamin D binding protein (DBP), fibroblast growth factor 23, soluble Klotho, sclerostin, copeptin, asymmetric dimethylarginine (ADMA) and urinary excretion of urea (as a measure of dietary protein intake).

The ViRTUE study offers the opportunity for post hoc studies investigating the effect of VDRA therapy in combination with dietary sodium restriction on various mineral-bone disease or cardiovascular parameters.

Statistical analysis and sample size

We will use standard descriptive statistics to assess baseline clinical and laboratory data at enrolment. Subsequently, we will compare albuminuria at the end of each study period by using mixed models repeated measures. Fixed factors will be sequence, period, medication (placebo or paricalcitol), diet (liberal or restricted sodium diet) and the interaction between medication and diet (medication x diet). The effect on blood pressure, renal function and other outcome parameters will be evaluated similarly. Patients who drop out during the study period will be analysed until the last hospital visit at which data has been collected, except for dropout due to screening failure.

Based upon data from a previous study[11], we calculated a sample size of 39 patients to detect a change of 23% in albuminuria (log delta albuminuria -0.26) with a power of 90%. If 25% dropout is taken into account, we require 50 patients. Of note, the sample size is smaller compared with a parallel study design, as subjects serve as their own internal control and the within-patient variability is smaller than the variability between patients.

Since no preliminary data exist on the effect of paricalcitol on ERPF, this will be assessed in a substudy which can be considered hypothesis-generating. We have enrolled 9 patients into the GFR/ERPF substudy.

Trial status

Patient enrolment started March 2012 and was closed in May 2014. The study will end in March 2015. First results from this trial are expected in the third quartile of 2015. The results will be presented at national and international scientific meetings. Publications will be submitted to peer-reviewed journals.

References

1. Jafar TH, Stark PC, Schmid CH, Landa M, Maschio G, Marcantoni C, et al. Proteinuria as a modifiable risk factor for the progression of non-diabetic renal disease. *Kidney Int* 2001; Sep;60(3):1131-40.

2. Hemmelgarn BR, Manns BJ, Lloyd A, James MT, Klarenbach S, Quinn RR, et al. Relation between kidney function, proteinuria, and adverse outcomes. *JAMA* 2010; Feb 3;303(5):423-9.

3. Maschio G, Alberti D, Janin G, Locatelli F, Mann JF, Motolese M, et al. Effect of the angiotensin-converting-enzyme inhibitor benazepril on the progression of chronic renal insufficiency. The Angiotensin-Converting-Enzyme Inhibition in Progressive Renal Insufficiency Study Group. *N Engl J Med* 1996; Apr 11;334(15):939-45.

4. Randomised placebo-controlled trial of effect of ramipril on decline in glomerular filtration rate and risk of terminal renal failure in proteinuric, non-diabetic nephropathy. The GISEN Group (Gruppo Italiano di Studi Epidemiologici in Nefrologia). *Lancet* 1997; Jun 28;349(9069):1857-63.

5. Apperloo AJ, de Zeeuw D, de Jong PE. Short-term antiproteinuric response to antihypertensive treatment predicts long-term GFR decline in patients with non-diabetic renal disease. *Kidney Int Suppl* 1994; Feb;45:S174-8.

6. Eijkelkamp WB, Zhang Z, Remuzzi G, Parving HH, Cooper ME, Keane WF, et al. Albuminuria is a target for renoprotective therapy independent from blood pressure in patients with type 2 diabetic nephropathy: post hoc analysis from the Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan (RENAAL) trial. *J Am Soc Nephrol* 2007; May;18(5):1540-6.

7. ONTARGET Investigators, Yusuf S, Teo KK, Pogue J, Dyal L, Copland I, et al. Telmisartan, ramipril, or both in patients at high risk for vascular events. *N Engl J Med* 2008; Apr 10;358(15):1547-59.

8. Parving HH, Brenner BM, McMurray JJ, de Zeeuw D, Haffner SM, Solomon SD, et al. Cardiorenal end points in a trial of aliskiren for type 2 diabetes. *N Engl J Med* 2012; Dec 6;367(23):2204-13.

9. Fried LF, Emanuele N, Zhang JH, Brophy M, Conner TA, Duckworth W, et al. Combined angiotensin inhibition for the treatment of diabetic nephropathy. *N Engl J Med* 2013; Nov 14;369(20):1892-903.

10. Heerspink HJ, Gao P, Zeeuw D, Clase C, Dagenais GR, Sleight P, et al. The effect of ramipril and telmisartan on serum potassium and its association with cardiovascular and renal events: results from the ONTARGET trial. *Eur J Prev Cardiol* 2014; Mar;21(3):299-309.

11. Vogt L, Waanders F, Boomsma F, de Zeeuw D, Navis G. Effects of dietary sodium and hydrochlorothiazide on the antiproteinuric efficacy of losartan. *J Am Soc Nephrol* 2008; May;19(5):999-1007.

12. Slagman MC, Waanders F, Hemmelder MH, Woittiez AJ, Janssen WM, Lambers Heerspink HJ, et al. Moderate dietary sodium restriction added to angiotensin converting enzyme inhibition compared with dual blockade in lowering proteinuria and blood pressure: randomised controlled trial. *BMJ* 2011; Jul 26;343:d4366.

13. Vegter S, Perna A, Postma MJ, Navis G, Remuzzi G, Ruggenenti P. Sodium intake, ACE inhibition, and progression to ESRD. *J Am Soc Nephrol* 2012; Jan;23(1):165-73.

14. Lambers Heerspink HJ, Holtkamp FA, Parving HH, Navis GJ, Lewis JB, Ritz E, et al. Moderation of dietary sodium potentiates the renal and cardiovascular protective effects of angiotensin receptor blockers. *Kidney Int* 2012; Aug;82(3):330-7.

15. Heeg JE, de Jong PE, van der Hem GK, de Zeeuw D. Efficacy and variability of the antiproteinuric effect of ACE inhibition by lisinopril. *Kidney Int* 1989; Aug;36(2):272-9.

16. Mirkovic K, van den Born J, Navis G, de Borst MH. Vitamin D in chronic kidney disease: new potential for intervention. *Curr Drug Targets* 2011; Jan;12(1):42-53.

17. de Borst MH, Hajhosseiny R, Tamez H, Wenger J, Thadhani R, Goldsmith DJ. Active vitamin D treatment for reduction of residual proteinuria: a systematic review. *J Am Soc Nephrol* 2013; Nov;24(11):1863-71.

18. Zhang Y, Kong J, Deb DK, Chang A, Li YC. Vitamin D receptor attenuates renal fibrosis by suppressing the renin-angiotensin system. *J Am Soc Nephrol* 2010; Jun;21(6):966-73.

19. Vaidya A, Forman JP, Hopkins PN, Seely EW, Williams JS. 25-Hydroxyvitamin D is associated with plasma renin activity and the pressor response to dietary sodium intake in Caucasians. *J Renin Angiotensin Aldosterone Syst* 2011; Sep;12(3):311-9.

20. de Borst MH, Vervloet MG, ter Wee PM, Navis G. Cross talk between the renin-angiotensin-aldosterone system and vitamin D-FGF-23-klotho in chronic kidney disease. *J Am Soc Nephrol* 2011; Sep;22(9):1603-9.

21. Yuan W, Pan W, Kong J, Zheng W, Szeto FL, Wong KE, et al. 1,25-dihydroxyvitamin D3 suppresses renin gene transcription by blocking the activity of the cyclic AMP response element in the renin gene promoter. *J Biol Chem* 2007; Oct 12;282(41):29821-30.

22. Ito I, Waku T, Aoki M, Abe R, Nagai Y, Watanabe T, et al. A nonclassical vitamin D receptor pathway suppresses renal fibrosis. *J Clin Invest* 2013; Nov 1;123(11):4579-94.

23. Zoccali C, Curatola G, Panuccio V, Tripepi R, Pizzini P, Versace M, et al. Paricalcitol and endothelial function in chronic kidney disease trial. *Hypertension* 2014; Nov;64(5):1005-11.

24. de Zeeuw D, Agarwal R, Amdahl M, Audhya P, Coyne D, Garimella T, et al. Selective vitamin D receptor activation with paricalcitol for reduction of albuminuria in patients with type 2 diabetes (VITAL study): a randomised controlled trial. *Lancet* 2010; Nov 6;376(9752):1543-51.

25. Arisz L, Donker AJ, Brentjens JR, van der Hem GK. The effect of indomethacin on proteinuria and kidney function in the nephrotic syndrome. *Acta Med Scand* 1976;199(1-2):121-5.

26. Laverman GD, Van Goor H, Henning RH, De Jong PE, De Zeeuw D, Navis G. Renoprotective effects of VPI versus ACEI in normotensive nephrotic rats on different sodium intakes. *Kidney Int* 2003; Jan;63(1):64-71.

27. Mirkovic K, Frenay AS, van den Born J, van Goor H, Navis GJ, de Borst MH. Renoprotective Effects of Vitamin D Receptor Agonist during Low but Not during High Dietary Sodium in Adriamycin Nephrosis [abstract] . *J Am Soc Nephrol* 2012;23:809A.

28. de Boer IH. Vitamin D and glucose metabolism in chronic kidney disease. *Curr Opin Nephrol Hypertens* 2008; Nov;17(6):566-72.

29. Thadhani R, Appelbaum E, Pritchett Y, Chang Y, Wenger J, Tamez H, et al. Vitamin D therapy and cardiac structure and function in patients with chronic kidney disease: the PRIMO randomized controlled trial. *JAMA* 2012; Feb 15;307(7):674-84.

30. Fishbane S, Chittineni H, Packman M, Dutka P, Ali N, Durie N. Oral paricalcitol in the treatment of patients with CKD and proteinuria: a randomized trial. *Am J Kidney Dis* 2009; Oct;54(4):647-52.

31. Alborzi P, Patel NA, Peterson C, Bills JE, Bekele DM, Bunaye Z, et al. Paricalcitol reduces albuminuria and inflammation in chronic kidney disease: a randomized double-blind pilot trial. *Hypertension* 2008; Aug;52(2):249-55.

32. Apperloo AJ, de Zeeuw D, Donker AJ, de Jong PE. Precision of glomerular filtration rate determinations for long-term slope calculations is improved by simultaneous infusion of 125I-iothalamate and 131I-hippuran. *J Am Soc Nephrol* 1996; Apr;7(4):567-72.

Chapter 7

THE EFFECT OF VITAMIN D RECEPTOR ACTIVATION AND DIETARY SODIUM RESTRICTION ON RESIDUAL ALBUMINURIA IN CHRONIC KIDNEY DISEASE: THE VIRTUE-CKD RANDOMIZED CONTROLLED TRIAL

Charlotte A. Keyzer[1], G. Fenna van Breda[2], Marc G. Vervloet[2], Maarten A. de Jong[1], Gozewijn D. Laverman[3], Marc H. Hemmelder[4], Wilbert M.T. Janssen[5], Hiddo J. Lambers Heerspink[6], Arjan J. Kwakernaak[1], Stephan J.L. Bakker[1], Gerjan Navis[1] and Martin H. de Borst[1] for the Holland Nephrology Study (HONEST) Network

1. Department of Internal Medicine, Division of Nephrology, University of Groningen, University Medical Centre Groningen, Hanzeplein 1, 9713 GZ, Groningen, the Netherlands;
2. Department of Nephrology and Institute for Cardiovascular Research, VU University Medical Centre, De Boelelaan 1117, 1081 HV, Amsterdam, the Netherlands;
3. Department of Internal Medicine, Division of Nephrology, ZGT Hospital, Zilvermeeuw 1, 7609 PP, Almelo, the Netherlands;
4. Department of Internal Medicine, Division of Nephrology, Medical Centre Leeuwarden, Henri Dunantweg 2, 8934 AD, Leeuwarden, the Netherlands;
5. Department of Internal Medicine, Division of Nephrology, Martini Hospital Groningen, Van Swietenplein 1, 9728 NT, Groningen, the Netherlands;
6. Department of Clinical Pharmacy and Pharmacology, University of Groningen, University Medical Centre Groningen, Hanzeplein 1, 9713 GZ, Groningen, the Netherlands

J Am Soc Nephrol 2017 apr; 28(4): 1296-1305. doi: 10.1681/ASN.2016040407.

Abstract

Reduction of residual albuminuria during single-agent RAAS-blockade is accompanied by improved cardio-renal outcomes in chronic kidney disease (CKD). We studied the individual and combined effects of the vitamin D receptor activator paricalcitol and dietary sodium restriction on residual albuminuria in CKD.

In a multi-center, randomized, placebo-controlled, cross-over trial 45 patients with non-diabetic CKD stage 1-3 and albuminuria >300 mg/24h despite ramipril 10 mg/d and blood pressure <140/90 mmHg were treated for four 8-week periods with paricalcitol (PARI, 2 µg/day) or placebo (PLAC), each combined with a low (LS) or regular sodium (RS) diet. The treatment effect was analyzed by linear mixed-effect models for repeated measurements.

In the *intention-to-treat* analysis, albuminuria was 1060 [778 to 1443] (geometric mean [95% CI]) mg/d during RS+PLAC, and 990 [755 to 1299] mg/24h during RS+PARI (P=0.2 vs. RS+PLAC). LS+PLAC reduced albuminuria to 717 [512 to 1005] mg/24h (P<0.001 vs. RS+PLAC), and LS+PARI to 683 [502 to 929] mg/24h (P<0.001 vs. RS+PLAC). The reduction by paricalcitol beyond the effect of LS was non-significant (P=0.6). In the *per protocol* analysis restricted to participants with ≥95% compliance to study medication, paricalcitol did provide further albuminuria reduction (P=0.04 LS+PARI vs. LS+PLAC). Dietary adherence was good, as reflected by urinary Na+ excretion of 174±64 mmol Na^+/day in the combined RS groups, and 108±61 mmol Na^+/day in the LS groups (P<0.001).

Moderate dietary sodium restriction substantially reduced residual albuminuria during fixed-dose ACEi. The additional effect of paricalcitol was small in comparison to sodium restriction and non-significant.

Introduction

Pharmacological renin-angiotensin-aldosterone system (RAAS)-blockade reduces albuminuria and blood pressure, subsequently retarding renal function loss and lowering the risk of cardiovascular morbidity and mortality in chronic kidney disease (CKD).[1-5] However, in a considerable proportion of patients, RAAS-blockade is unable to halt the progression of CKD, despite blood pressure control. Residual albuminuria (or proteinuria), persisting despite optimally dosed RAAS-blockade, is strongly associated with adverse long-term renal and cardiovascular outcomes,[6,7] and therefore considered a target for additional intervention.

Dietary sodium restriction potentiates the albuminuria-lowering efficacy of RAAS-blockade in non-diabetic and diabetic CKD patients,[8-10] which has been associated with improved long-term cardiorenal protection.[11,12] In addition, vitamin D receptor activator (VDRA) therapy may lower residual albuminuria, as suggested by preclinical studies[13,14] and several small-to-medium-scale randomized controlled trials in CKD patients.[15,16] The renoprotective effect of VDRA therapy may at least in part be mediated by a direct inhibitory effect on the RAAS.[17,18] Given the consistent finding that dietary sodium restriction potentiates the albuminuria-lowering efficacy of conventional RAAS-blockade including angiotensin converting enzyme inhibition (ACEi)[9] and angiotensin receptor blockade,[8] it seems plausible that sodium restriction would also potentiate the capacity of VDRA treatment to lower residual albuminuria. In line with this assumption, we recently found that dietary sodium restriction potentiates the antiproteinuric and renoprotective efficacy of VDRA treatment in a rat model of proteinuric nephropathy,[19] and that sodium intake modulates the inverse association between plasma vitamin D levels and the risk of developing increased albuminuria in the general population.[20] At variance, however, a post-hoc analysis of the VITAL trial[16] as well as an observational study in non-diabetic CKD[21] suggested that albuminuria patients with higher baseline dietary sodium intake had a stronger antiproteinuric response to VDRA treatment than those with lower baseline sodium intake.

In the ViRTUE-CKD trial, therefore, we prospectively studied the separate and combined albuminuria-lowering effect of the VDRA paricalcitol and dietary sodium restriction during fixed-dose RAAS-blockade, the current standard treatment, in non-diabetic patients with CKD. The trial compares residual albuminuria during four subsequent study periods in random order: paricalcitol or placebo combined with either dietary sodium restriction (target 50 mmol Na^+/day) or a regular sodium diet (target 200 mmol Na^+/day), respectively, all during fixed-dose ACEi.

Concise methods

Study design

We performed an investigator-initiated, multicenter, randomized, double-blind, placebo-controlled cross-over trial in five Dutch hospitals. Patients were included between January 2012 and May 2014. Inclusion was concluded upon reaching the predefined sample size (see below); the last follow-up visit of the last patient took place in March 2015. The study was conducted according to the principles of the Declaration of Helsinki; the study protocol has been approved by the Medical Ethical Committee of the University Medical Centre Groningen, the Netherlands (METc 2009.272) and has been registered in the Dutch clinical trial register (NTR2898). The rationale and study protocol of the ViRTUE-CKD study have been published previously.[45]

Participants

We recruited stage 1–3 non-diabetic patients with CKD (creatinine clearance >30 mL/min) and residual albuminuria. Inclusion criteria were: residual albuminuria >300 mg/day despite single-agent RAAS blockade, stable renal function (<6 mL/min decline in the previous year) with a creatinine clearance >30 mL/min, parathyroid hormone (PTH) values <1.5 times the upper limit of normal, serum calcium (adjusted for serum albumin) between 2.0 and 2.6 mmol/L, serum phosphate ≤1.5 mmol/L, and age over 18 years. Exclusion criteria were: diabetes mellitus, uncontrolled hypertension, hyperkalemia (potassium >6.0 mmol/L), a cardiovascular event in the previous six months, heart failure NYHA III-IV, epilepsy, liver disease, active malignancy, a bowel disorder resulting in fat malabsorption, treatment with vitamin D analogue in the previous three months, regular use (>2 doses/week) of non-steroidal anti-inflammatory drugs, use of immunosuppressive treatment, digoxin or psychiatric medication, drug or alcohol abuse, incompliance with the study diet or study medication, pregnancy, or breast feeding.

Study design

Detailed information regarding the study protocol has been published previously.[45] During a run-in period, patients received standardized RAAS-blockade (10 mg ramipril/day). Existing treatment with other RAAS-blocking agents and diuretics (except for furosemide) was discontinued. If the target blood pressure of <140/90 mmHg was not reached within 6 weeks after the initiation of ramipril, additional antihypertensive therapy (metoprolol, doxazosin and/or amlodipine) was added to the treatment regimen with 4-week intervals. When the target blood pressure was reached, patients were allowed to enter the study protocol. After a maximum wash-in/wash-out period of 18 weeks, patients with a blood pressure value <180/100 mmHg were able to enroll in the study, whereas patients with a blood pressure >180/100 mmHg were not included in the study.[45]

Patients were subjected to four subsequent treatment periods of eight weeks each. These study periods consisted of (i) the VDRA paricalcitol (19-nor-1,25[OH]$_2$–vitamin D2, 2 µg/day) combined with a regular sodium diet (target sodium intake 200 mmol Na^+/day [≈4.8 g], i.e. the average sodium intake in the general population); (ii) paricalcitol (2 µg/day) combined with dietary sodium restriction (target sodium intake 50 mmol Na+/day, [≈1.2 g]), (iii) placebo combined with a regular sodium diet or (iv) placebo combined with dietary sodium restriction. To prevent systematic errors resulting from the cross-over design, the order of the treatment periods was randomized (1:1:1:1) for each patient. Four different treatment sequences were defined.[45] The study medication (paricalcitol or placebo) was provided by AbbVie. Placebo capsules had a similar appearance, smell and taste as compared with paricalcitol capsules. Computer-generated randomization was performed by AbbVie. The investigators (CAK and GFvB) enrolled participants. Patients received study medication containers labelled with a unique number representing the randomly allocated sequence, whereby all participants and involved investigators and care providers remained blinded to the study medication type (paricalcitol or placebo) throughout the entire study. Assignment of the treatment order was not disclosed until the study database was locked. The dietary intervention was open label.

At the start of the first dietary sodium restriction study period, patients received personal dietary advice from a dietician. Sodium restriction was achieved by replacing sodium-rich products with a low sodium product of the same product group, aiming for isocaloric intake with a similar balance among protein, carbohydrate and fat. Compliance to the sodium diet was monitored by measuring 24-hour urinary sodium excretion every 4 weeks, and patients were counselled to use this information.

At start of the run-in period, medication use of the participants was verified. Use of (self-initiated) vitamin supplementation was specifically inquired. Any form of vitamin D supplementation was discontinued. Participants were instructed not to use supplemental vitamin D (calciferol) and to report all changes in prescribed and self-initiated medication use during the entire study.

Four weeks after the start of each treatment period, serum albumin, calcium and PTH were measured for a safety analysis. In case of hypercalcemia (corrected serum calcium >2.60 mmol/L) or hypoparathyroidism (PTH <1.5 pmol/L), the dose of the study medication (paricalcitol or placebo) was reduced from two capsules to one capsule per day for the remaining study period(s). All patient-reported or observed adverse effects were recorded.

Primary and secondary endpoints

The primary end point of our study was albuminuria, measured in a 24-hour urine sample collected at the end of each study period. Secondary study endpoints were blood pressure, creatinine clearance, eGFR, urinary sodium excretion and plasma renin concentration, respectively, measured at the end of each study period.

Measurements

At the end of each 8-week treatment period, patients collected 24-hour urine samples, blood pressure was measured, and a blood sample was taken after overnight fasting. Albuminuria was measured using a turbidimetric assay using benzethonium chloride (Modular, Roche Diagnostics, Mannheim, Germany). Blood pressure was evaluated during every outpatient clinic visit under constant conditions, at one-minute intervals for 15 min by an automatic device (Dinamap; DE Medical systems, Milwaukee, WI), with the patient in a semi-supine position. The mean of three readings was used for further analysis.[45] Blood electrolytes, lipids, proteins, and urinary electrolytes were determined by using an automated multi-analyzer (Modular, Roche Diagnostics, Mannheim, Germany). Plasma renin concentration was measured using a 2-site immunoradiometric assay (Beckman Coulter, Immunotech, Prague, Czech Republic). Parathyroid hormone (PTH) concentrations were assessed with the Roche Cobas electrochemoluminescent immunometric assay (Roche Diagnostics, Mannheim, Germany). 25-hydroxyvitaminvitamin D3 (25[OH]D) levels were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS). Carboxyl-terminal FGF23 was determined in duplicate using a human FGF23 ELISA (Immutopics, San Clemente, CA, USA).

Dietary sodium intake was assessed from urinary sodium excretion in 24-hour urine samples. Creatinine clearance was calculated from creatinine concentrations in plasma and in 24-hour urine collections, and eGFR was calculated using the creatinine-based CKD Epidemiology Collaboration (EPI) formula.[46] Serum calcium was adjusted for hypoalbuminemia as follows: corrected calcium = serum calcium (mmol/L) + 0.023 * (40 – serum albumin [g/L]) if serum albumin < 35 g/L. Peripheral pitting edema was assessed at the pre-tibia area of both legs by visual and manual examination and scored dichotomously (absent or present).

Statistical analysis

Based upon data from a previous study,[8] a sample size of 39 patients was calculated to detect a difference of 23% in albuminuria (log delta albuminuria –0.26) between HS+placebo and LS+paricalcitol with 90% power, considering a standard deviation of 0.5 for the log delta albuminuria.[45] Assuming a dropout rate of 15%, we aimed to include 45 patients. The sample

size calculation took into account that each patient serves as his own internal control, increasing statistical power.

Data are presented as mean ± SD in case of normally distributed data, geometric mean [95% confidence interval] for non-normally distributed data, and number (percentage) for nominal data, unless stated otherwise. The relative change in albuminuria between study periods is presented as median [interquartile range]. Variable distribution was tested with histograms and probability plots. *P* for differences between the four treatment sequences were assessed with ANOVA for normally distributed continuous data, Kruskal-Wallis test for non-normally distributed data, and the $\chi 2$ test for nominal data. Data at the end of the run-in period were considered baseline values. To determine the effect of treatment, we used linear mixed-effect models for repeated measurements, using the unstructured covariance structure with random intercept, and 'center', 'treatment' and 'sequence' as well as their interaction ('treatment*sequence') as fixed factors. Non-normally distributed variables were 2log transformed before entering the model. Linear mixed model analysis was used to investigate possible carryover effects: non-significant ($P>0.05$) effects of sequence and treatment*sequence were interpreted as indicating that carryover effects were absent.

To investigate a possible interaction between the interventions on the primary endpoint we also analyzed the primary outcome (2log transformed albuminuria) by linear mixed-effect models for repeated measurements, using the unstructured covariance structure with random intercept, and 'center', 'period', 'sequence', 'medication' (placebo or paricalcitol) and 'diet' (normal or low sodium diet) as well as their interaction ('medication'*'diet') as fixed factors.

For the primary analysis, all available data from all 45 patients were included (*intention-to-treat* analysis). As a *per protocol* analysis, we re-analyzed the primary endpoint in a study population restricted to those participants with ≥95% compliance to the study medication (assessed by counting the returned paricalcitol capsules), for each treatment period. There were 31-34 participants available for this analysis. To account for missing data, we report the estimated (geometric) means obtained from the linear mixed modelling for this analysis. In another secondary analysis we addressed the compliance to the dietary sodium restriction by adding 24h urinary Na^+ excretion (as a continuous variable) to the linear mixed model analysis.

A two-tailed $P<0.05$ was considered to indicate statistical significance. Statistical analyses were performed using SPSS 22.0 for Windows (IBM SPSS, Chicago, IL) and GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

Results

Study population

Of 212 eligible patients, 68 patients gave written informed consent and were subsequently enrolled in the run-in period in which blood pressure was targeted <140/90 mmHg using a standardized regimen (Figure 1). During the run-in period, 23 patients discontinued the study. Of the 45 patients subsequently randomized, three patients were excluded during the study after completion of at least one study period. Supplemental Table 1 shows the baseline characteristics of the 45 study participants after randomization, according to the sequence of the study periods. All patients received background ACEi in a fixed dose throughout the study (ramipril 10 mg/d).

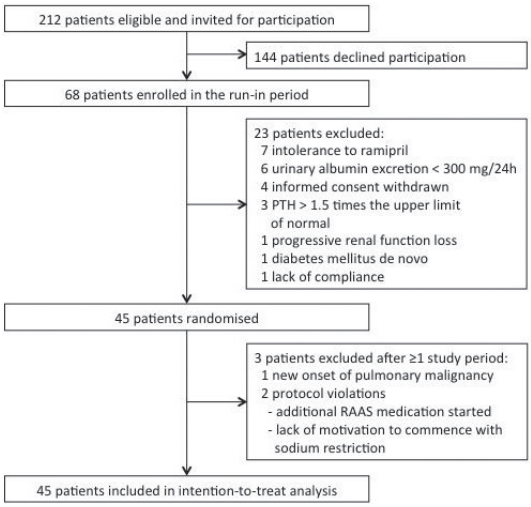


Figure 1. Trial profile of the ViRTUE-CKD study.

Primary efficacy analysis

During regular sodium (RS) diet combined with placebo treatment, residual albuminuria was 1,060 [778 to 1,443] mg/24h, Figure 2). In the *intention to treat* analysis, paricalcitol provided a weak and non-significant albuminuria reduction to 990 [755 to 1,299] mg/24h (-12.5% [-26.0% to 26.3%] vs. RS+placebo, *P*=0.2; Figure 3). During a low sodium (LS) diet combined with placebo treatment, albuminuria was reduced to 717 [512 to 1,005] mg/24h (-25.4% [-52.6% to -2.3%] vs. RS+placebo, *P*<0.001). The strongest albuminuria reduction was reached by LS+paricalcitol, to 683 [502 to 929] mg/24h (-31.7% [-55.0% to -0.9%], *P*<0.001 vs. RS+placebo). Paricalcitol did not reduce albuminuria further than the effect of the LS diet in itself (*P*=0.6). Adjustment for blood pressure did not change the results. Results were similar for the urinary albumin/creatinine ratio (Table 1). Linear mixed-effect model analysis indicated no carryover effects (center *P*=0.7, treatment *P*<0.001, sequence *P*=0.9 and

treatment*sequence *P*=0.4). Linear mixed-effect model analysis also indicated no interaction between the two interventions (center *P*=0.6, period *P*=0.3, sequence *P*=0.7, medication *P*=0.3, diet *P*<0.001 and medication*diet *P*=0.8). During RS+placebo albuminuria and 25(OH)D were not significantly correlated (linear regression β=-0.02, P=0.1). The albuminuria lowering effect of dietary sodium restriction and paricalcitol was not influenced by the level of 25(OH)D (linear mixed-effect model analysis P=0.5).

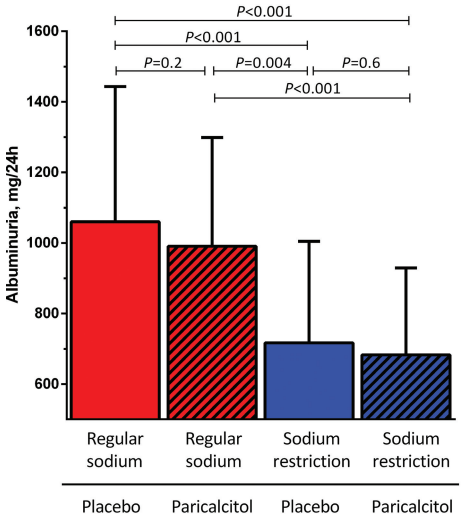


Figure 2. Effect of sodium restriction and paricalcitol on albuminuria in the *intention-to-treat* analysis. Albuminuria during regular sodium diet or dietary sodium restriction in combination with paricalcitol (2 microgram/day) or placebo. Data is shown as geometric mean (95% CI). *P* value shows treatment effect by linear mixed modelling with center, treatment, sequence and the interaction treatment*sequence as fixed factors.

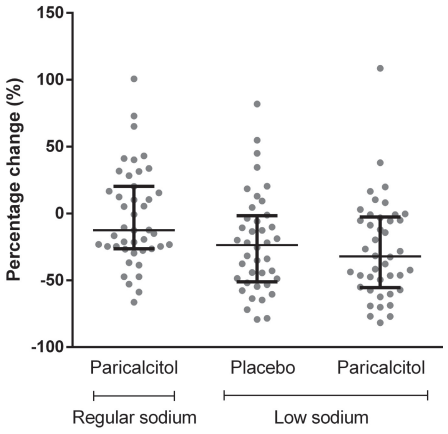


Figure 3. Relative change in residual albuminuria compared to RS+placebo in the intention-to-treat analysis. The percentage change is shown as individual data with median and interquartile range. Data for one participant with extreme values (+259%, -61% and +165%, respectively) are not shown.

Secondary and exploratory outcomes

Mean arterial pressure (MAP) was 95 [92 to 98] mmHg during RS+placebo (Figure 4). Paricalcitol did not affect MAP, neither during RS diet (95 [91 to 98] mmHg; *P*=0.8 vs RS+placebo), nor during LS diet (90 [87 to 93] mmHg; *P*=0.5 vs. LS+placebo). Dietary sodium restriction in itself reduced MAP to 90 [88 to 93] mmHg (*P*<0.001 LS+placebo vs RS+placebo). Treatment effects were similar for systolic and diastolic blood pressure (Table 1).

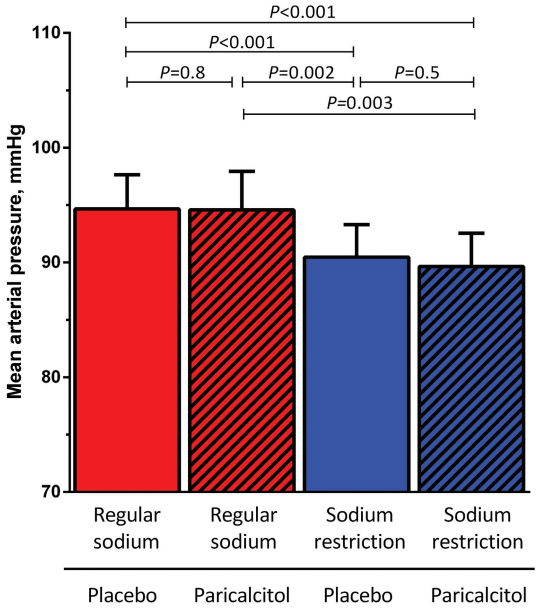


Figure 4. Effect of sodium restriction and paricalcitol on blood pressure in the *intention-to-treat* analysis. Mean arterial pressure during regular sodium diet or dietary sodium restriction in combination with paricalcitol (2 microgram/day) or placebo. Data is shown as mean (95% CI). *P* value shows treatment effect by linear mixed modelling with center, treatment, sequence and the interaction treatment*sequence as fixed factors.

Creatinine clearance was 101±41 mL/min during RS+placebo (Table 1), and was not significantly changed by paricalcitol (97±38 mL/min; *P*=0.2). Sodium restriction induced a reduction in creatinine clearance, both during placebo (91±38 mL/min; *P*=0.01 vs. RS+placebo) and during paricalcitol (90±35 mL/min; *P*=0.004). Paricalcitol did not influence creatinine clearance beyond the effect of dietary sodium restriction (*P*=0.7 vs. LS+placebo).

Both during RS and during LS diet, paricalcitol increased serum phosphate and urinary calcium excretion and reduced parathyroid hormone (PTH), consistent with the known effects of paricalcitol on calcium and phosphate metabolism (Table 1). During dietary sodium restriction, paricalcitol also increased serum calcium. Dietary sodium restriction decreased body weight

and plasma sodium, and increased plasma renin and albumin concentrations, consistent with a reduction of extracellular volume. Paricalcitol did not affect these parameters (Table 1). Both LS diet and paricalcitol increased plasma carboxy-terminal fibroblast growth factor 23 (FGF-23; Table 1). Serum 25(OH)D was not affected by LS diet or by paricalcitol (Table 1).

Adverse effects

Nine patients developed hypercalcemia during a paricalcitol treatment period, and in five of these patients hypercalcemia was also present during a placebo treatment period. From these five patients two developed hypercalcemia during a placebo treatment period before having received paricalcitol treatment, and the other three had at least one normal calcium measurement between hypercalcemia during a paricalcitol treatment period and hypercalcemia during a placebo treatment. There was no persisting hypercalcemia when paricalcitol was ceased. Hypercalcemia during a safety control visit led to a dose reduction in five patients: two during RS+paricalcitol and three during LS+paricalcitol. Severe orthostatic complaints required tapering of antihypertensive medication in one patient during LS+paricalcitol. Mild orthostatic complaints, not necessitating drug withdrawal, occurred in two patients on RS+placebo and one patient on paricalcitol+RS, and in ten patients on LS+placebo and four patients on LS+paricalcitol. These and all other reported adverse effects possibly or probably related to treatment are listed in Supplemental Table 2.

Compliance

We assessed compliance to the diet by 24-hour urinary sodium excretion and compliance to study medication from counting returned capsules. Mean urinary sodium excretion was 174 ± 64 mmol Na+/day (approximately 4000 mg Na^+/day or 10 g NaCl/day) during the two study periods on the RS diet, and 108 ± 61 mmol Na^+/day (approximately 2500 mg Na^+/day or 6.2 g NaCl/day; $P<0.001$ vs. RS diet) during the two LS periods. Compliance to the pharmacological intervention was similar among the four treatment periods (Table 1).

Per protocol analysis

The primary endpoint was re-analyzed in participants with ≥95% compliance to the study medication, assessed per study period. For each study period, data from 31-34 participants were available for this analysis. Compliance of the excluded participants during the excluded study periods was 88±7%, and unknown for 5 patients (during 10 study periods). Supplemental Table 3 shows the main clinical parameters during the four treatment periods of participants in the *per protocol* analysis. Here, estimated albuminuria was 1,177 [823 to 1,682] mg/24h during RS+placebo. During RS diet, paricalcitol provided a non-significant albuminuria reduction to 1,082 [772 to 1,516] mg/24h (-18.0% [-27.0% to 29.1%]; $P=0.3$ vs. RS+placebo). In contrast, dietary

Table 1. Clinical parameters during the four treatment periods; *intention-to-treat* analysis

	Regular sodium diet		Sodium restriction diet	
	Placebo	Paricalcitol	Placebo	Paricalcitol
	N= 44	N= 44	N= 43	N= 43
Plasma/Serum				
Hb, mmol/L	9.0 ± 0.9	9.0 ± 0.8	9.1 ± 0.9	9.0 ± 0.8
Sodium, mmol/L	140.6 ± 2.3	140.1 ± 2.0	139.8 ± 2.4*	140.4 ± 2.4‡
Potassium, mmol/L	4.3 ± 0.4	4.2 ± 0.4	4.3 ± 0.4†	4.4 ± 0.5†
Calcium, mmol/L	2.35 ± 0.11	2.37 ± 0.10	2.37 ± 0.13	2.41 ± 0.15*‡
Phosphate, mmol/L	0.94 ± 0.17	0.98 ± 0.16*	0.94 ± 0.14	1.00 ± 0.15*‡
Creatinine, µmol/L	110 ± 32	112 ± 32	113 ± 31	120 ± 35*†‡
eGFR, ml/min/1.73m^2	68 ± 25	67 ± 24	67 ± 24	63 ± 25*†‡
Albumin, g/L	38 ± 5	39 ± 5	40 ± 4*	40 ± 4*
Total cholesterol, mmol/L	5.2 ± 1.2	5.2 ± 1.2	4.9 ± 1.0*†	5.1 ± 1.2‡
HDL cholesterol, mmol/L	1.4 ± 0.4	1.4 ± 0.4	1.3 ± 0.4*†	1.3 ± 0.4
LDL cholesterol, mmol/L	3.1 ± 0.9	3.0 ± 1.0	2.9 ± 0.7*	3.1 ± 0.90
Renin, pg/mL	42.9 [30.9-59.5]	45.3 [32.5-63.1]	61.3 [44.6-84.2]*†	66.5 [48.4-91.4]*†
PTH, pmol/L	5.0 [4.4-5.7]	3.5 [3.0-4.1]*	5.5 [4.8-6.2]†	3.4 [3.0-4.0]*‡
25(OH)D, nmol/L	50.4 ± 22.8	50.6 ± 23.4	52.7 ± 22.6	56.4 ± 24.2
FGF23, RU/mL	114 [102-128]	139 [122-158]*	120 [106-135]*†	152 [130-178] *†‡
Urine				
Creatinine, mmol/24h	14.7 ± 3.9	14.5 ± 3.8	13.8 ± 3.7*	14.4 ± 3.4‡
Sodium, mmol/24h	170 ± 61	178 ± 68	104 ± 59*†	111 ± 63*†
Urea, mmol/24h	419 ± 128	416 ± 132	383 ± 120*	404 ± 118
Potassium, mmol/24h	78 ± 25	80 ± 25	81 ± 26	82 ± 24
Calcium, mmol/24h	2.4 ± 2.0	4.5 ± 3.3*	2.2 ± 2.4†	3.9 ± 2.9*‡
Phosphate, mmol/24h	32.4 ± 9.5	33.8 ± 13.3	30.4 ± 13.2	31.5 ± 9.9
Albuminuria mg/24h	1,060 [778-1,443]	990 [755-1,299]	717 [512-1,005]	683 [502-929]
Proteinuria, g/24h	1.4 [1.0-1.8]	1.3 [1.0-1.6]	1.0 [0.7-1.3]*†	0.9 [0.7-1.2]*†
Albumin/creatinine ratio	75 [55-101]	71 [53-94]	54 [39-75]*†	49 [36-66]*†
Creatinine clearance, mL/min	101 ± 41	97 ± 38	91 ± 38*	90 ± 35*
Other				
Systolic blood pressure, mmHg	129 ± 14	128 ± 14	123 ± 12*†	122 ± 12*†
Diastolic blood pressure, mmHg	77 ± 9	78 ± 11	74 ± 9*†	74 ± 9*†
Mean arterial pressure, mmHg	95 ± 10	95 ± 11	90 ± 9*†	90 ± 9*†
Heart rate, bpm	65 ± 10	66 ± 10	65 ± 10	65 ±10
Body weight, kg	90 ± 17	89 ± 17	88 ± 18*†	87 ± 17*†
Compliance, %	95 ± 7	97 ± 6	97 ± 5	97 ± 4

Data are presented as mean ± SD or geometric mean [95% CI] for normally or skewed distributed data, respectively. *P* value shows treatment effect by linear mixed modelling with center, treatment, sequence and the interaction treatment*sequence as fixed factors.

*P< 0.05 versus placebo on regular sodium diet

†P<0.05 versus paricalcitol on regular sodium diet

‡P<0.05 versus placebo on sodium restriction diet

sodium restriction in itself reduced albuminuria to 804 [564 to 1,146] mg/24h (-35.1% [-53.9% to -6.8%]; *P*<0.001 vs. RS+placebo), and the combination of paricalcitol and dietary sodium restriction further reduced albuminuria to 690 [480 to 993] mg/24h (-42.0% [-59.6% to -5.9%]; *P*=0.04 vs. RS+placebo). In this analysis, paricalcitol significantly reduced albuminuria beyond the effect of sodium restriction (*P*=0.04 LS+paricalcitol vs. LS+placebo). Similar results were observed when considering the urinary albumin/creatinine ratio (Supplemental Table 3). There was no interaction between the two interventions on the primary endpoint (center P=0.8, period P=0.2, sequence P=0.9, medication P=0.03, diet P<0.001 and medication*diet P=0.3).

During regular sodium diet, but not during sodium restriction, paricalcitol treatment resulted in a small but significant reduction in MAP (*P*=0.045; Supplemental Table 3). Additional adjustment for urinary sodium excretion did not materially influence the results on residual albuminuria, but the effect of paricalcitol during regular sodium intake on MAP was no longer significant (*P*=0.07).

Discussion

The main aim of this trial was to prospectively study the separate and combined effect of paricalcitol and dietary sodium restriction to lower residual albuminuria during fixed-dose single-agent RAAS-blockade in non-diabetic patients with CKD. Moderate dietary sodium restriction substantially reduced residual albuminuria, whereas the effect of paricalcitol was non-significant. There was no interaction between the dietary sodium intake and paricalcitol on albuminuria reduction. Our prospective data did not confirm the previously raised suggestion that albuminuria reduction by paricalcitol is optimal during high sodium intake.[16,21]

The capacity of paricalcitol to reduce albuminuria or proteinuria has been suggested in several clinical studies in different CKD populations, predominantly, albeit not exclusively in diabetic patients.[16,21-26] Two previously published reports based on post-hoc analyses from clinical studies suggested that paricalcitol provides stronger albuminuria reduction in patients with higher baseline sodium intake.[16,21] This was interpreted as related to suboptimal RAAS-blockade efficacy during high sodium intake,[21] and consequently paricalcitol was suggested to be a suitable add-on to RAAS-blockade for patients on high sodium intake.[16] Our prospective intervention is at variance with the latter suggestion.

Our results are consistent with several clinical studies showing that sodium intake potentiates RAAS-blockade,[8-10,27,28] as well as with recent data from a prospective study in a rat model of proteinuric nephropathy[19]. In this study combined treatment with paricalcitol and an ACEi reduced

proteinuria, renal interstitial inflammation, glomulerosclerosis and interstitial prefibrotic changes during low sodium, but not during high sodium intake.[19] Dietary sodium restriction reduced residual albuminuria and blood pressure during single-agent RAAS-blockade, in line with previous studies.[8-10] Paricalcitol in itself provided only a mild further reduction of residual albuminuria beyond dietary sodium restriction, in contrast with prior findings with hydrochlorothiazide, which further reduced residual proteinuria beyond the effect of sodium restriction and angiotensin receptor blockade in a previous study.[8] The effect of paricalcitol added to sodium restriction was stronger and reached statistical significance in a *per protocol* analysis restricted to patients with >95% compliance to study medication. A possible explanation for the relatively small effect of paricalcitol on albuminuria during ACEi and sodium restriction could be the substantially lower albuminuria elicited by sodium restriction in itself. Residual proteinuria during sodium restriction was relatively low compared with other trials in non-diabetic CKD patients treated with paricalcitol,[21,24] suggesting that the efficacy of ACEi combined with LS diet may have diluted the residual treatment effect of paricalcitol. Furthermore, it should also be taken into consideration that our study had a run-in period to optimize RAAS-blockade and antihypertensive treatment, because we were interested in the effect of add-on paricalcitol on residual albuminuria during optimal treatment. The albuminuria-lowering effect of paricalcitol was not influenced by the baseline 25(OH)D level; therefore pre-existent vitamin D status is unlikely to explain the non-significant effect of paricalcitol.

The renoprotective effects of moderate sodium restriction during single RAAS-blockade, lowering albuminuria and blood pressure, are likely multifactorial. The capacity of sodium restriction to reduce residual albuminuria is probably not only mediated by blood pressure,[8] but additionally by anti-inflammatory and anti-fibrotic pathways,[29-31] and local tissue RAAS activity in kidney, vasculature and brain.[32]

Experimental studies have shown that VDRA treatment exerts direct protective effects on podocytes,[33] negatively regulates the RAAS by suppressing renin production,[17,34,35] and has anti-inflammatory and anti-fibrotic effects.[13,36,37] These effects could either alone or, most likely; in combination, explain the anti-albuminuric effect of VDRA in addition to RAAS-blockade, as also supported by our recent preclinical data, showing renal tissue protection during ACEi, paricalcitol and dietary sodium restriction in experimental proteinuric nephropathy.[19] In our trial in non-diabetic CKD patients, the *intention to treat* analysis showed no additional albuminuria lowering effect of paricalcitol on top of the dietary sodium restriction. Our results are in line with recent studies in non-diabetic CKD[25,38,39] where the anti-albuminuric effect of paricalcitol was less than expected based on studies in diabetic CKD, or even absent. In the absence of head-to-head

comparisons between diabetic and non-diabetic CKD however, it remains unclear whether there is a consistent difference in responsiveness to paricalcitol between diabetic and non-diabetic patients. The absence of an antihypertensive effect of paricalcitol is in accordance with a recent meta-analysis showing that neither paricalcitol nor other vitamin D analogues are effective in lowering blood pressure.[40]

Both sodium restriction and paricalcitol were well tolerated. The most common adverse effects were mildly symptomatic hypotension (sodium restriction) and hypercalcemia (paricalcitol). Creatinine clearance was significantly reduced by the dietary sodium restriction. This decline was reversible and therefore probably reflects a reduction of glomerular pressure. It has been shown that a reduction in renal function during initiation of RAAS-blockade predicts a slower rate of long-term renal function decline.[41,42] These data suggest that the initial fall in renal function in response to antihypertensive therapy reflects renal protection, but whether this is also true for the effect of dietary sodium restriction on top of RAAS-blockade has not been established. Paricalcitol also increased serum creatinine and (consequently) decreased creatinine-based eGFR, creatinine clearance was not influenced by paricalcitol treatment on either sodium intake. An increase in serum creatinine without altering the true GFR has been reported previously for paricalcitol,[43] and may be related to an effect on muscle metabolism.

Whether the combination of paricalcitol and dietary sodium restriction translates into beneficial long-term outcomes remains to be addressed, but caution is warranted in extrapolation from anti-albuminuric effects only, as potential beneficial effects of the lower albuminuria could be counterbalanced by unfavorable effects of the rise in serum phosphate and the phosphate-regulating hormone FGF23 triggered by paricalcitol.[44] To investigate the overall effect of VDRA treatment, combined with moderate sodium restriction, on long-term clinical outcomes in CKD, a large randomized controlled clinical trial would be needed.

A limitation of our study is the limited exposure time to paricalcitol, precluding conclusions on the effect of paricalcitol and dietary sodium restriction on long-term clinical outcomes. The length of treatment periods was based on previous studies with paricalcitol demonstrating maximum albuminuria reduction at 4-6 weeks after treatment initiation.[16, 23] No washout periods were included in the study design; the 8-week period was long enough to minimize potential carryover. Furthermore, the sample size is relatively small, which increases the chance of a false negative finding; on the other hand, the cross-over design increased statistical power as subjects served as their own internal control and the within-patient variability is smaller than the variability between patients. Third, our study was performed in a selection of highly motivated patients under well-

controlled and intensive treatment, limiting the external validity of our findings. Because we aimed to study the effect of sodium restriction in a clinically relevant set up, we applied sodium intervention by dietary counseling, rather than in a blinded design with add-on placebo or sodium supplement. Lastly, blood pressure was evaluated during outpatient clinic visits for 15 min by an automatic device and not by 24-hour ambulatory blood pressure monitoring. On the other hand, major strengths of our study include the cross-over design with participants serving as their own internal control, the documentation of sodium intake by 24-hour urinary excretion and the prospective intervention design to investigate the influence of sodium intake on the renoprotective efficacy of add-on paricalcitol.

In conclusion, moderate dietary sodium restriction strongly and significantly reduced residual albuminuria during single-agent RAAS-blockade. Furthermore, paricalcitol had a small, non-significant effect on reducing residual albuminuria in non-diabetic patients with CKD. In this prospective study we did not confirm that albuminuria reduction by paricalcitol is optimal during high sodium intake; oppositely, there was a trend towards optimal albuminuria reduction of paricalcitol during sodium restriction. The capacity of moderate sodium restriction to potentiate the antiproteinuric effect of conventional RAAS-blockade has been associated with cardiorenal protection in both diabetic[12] and non-diabetic[11] CKD. Future studies should address whether the combination of paricalcitol and dietary sodium restriction may further enhance cardiorenal protection in addition to conventional RAAS-blockade.

References

1. Zucchelli P, Zuccala A, Borghi M, Fusaroli M, Sasdelli M, Stallone C, Sanna G, Gaggi R: Long-term comparison between captopril and nifedipine in the progression of renal insufficiency. *Kidney Int* 42: 452-458, 1992

2. Maschio G, Alberti D, Janin G, Locatelli F, Mann JF, Motolese M, Ponticelli C, Ritz E, Zucchelli P: Effect of the angiotensin-converting-enzyme inhibitor benazepril on the progression of chronic renal insufficiency. the angiotensin-converting-enzyme inhibition in progressive renal insufficiency study group. *N Engl J Med* 334: 939-945, 1996

3. Randomised placebo-controlled trial of effect of ramipril on decline in glomerular filtration rate and risk of terminal renal failure in proteinuric, non-diabetic nephropathy. the GISEN group (gruppo italiano di studi epidemiologici in nefrologia). *Lancet* 349: 1857-1863, 1997

4. Yusuf S, Sleight P, Pogue J, Bosch J, Davies R, Dagenais G: Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. the heart outcomes prevention evaluation study investigators. *N Engl J Med* 342: 145-153, 2000

5. Brenner BM, Cooper ME, de Zeeuw D, Keane WF, Mitch WE, Parving HH, Remuzzi G, Snapinn SM, Zhang Z, Shahinfar S, RENAAL Study Investigators: Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. *N Engl J Med* 345: 861-869, 2001

6. Ruggenenti P, Perna A, Remuzzi G, GISEN Group Investigators: Retarding progression of chronic renal disease: The neglected issue of residual proteinuria. *Kidney Int* 63: 2254-2261, 2003

7. Holtkamp FA, de Zeeuw D, de Graeff PA, Laverman GD, Berl T, Remuzzi G, Packham D, Lewis JB, Parving HH, Lambers Heerspink HJ: Albuminuria and blood pressure, independent targets for cardioprotective therapy in patients with diabetes and nephropathy: A post hoc analysis of the combined RENAAL and IDNT trials. *Eur Heart J* 32: 1493-1499, 2011

8. Vogt L, Waanders F, Boomsma F, de Zeeuw D, Navis G: Effects of dietary sodium and hydrochlorothiazide on the antiproteinuric efficacy of losartan. *J Am Soc Nephrol* 19: 999-1007, 2008

9. Slagman MC, Waanders F, Hemmelder MH, Woittiez AJ, Janssen WM, Lambers Heerspink HJ, Navis G, Laverman GD, HOlland NEphrology STudy Group: Moderate dietary sodium restriction added to angiotensin converting enzyme inhibition compared with dual blockade in lowering proteinuria and blood pressure: Randomised controlled trial. *BMJ* 343: d4366, 2011

10. Kwakernaak AJ, Krikken JA, Binnenmars SH, Visser FW, Hemmelder MH, Woittiez AJ, Groen H, Laverman GD, Navis G, Holland Nephrology Study (HONEST) Group: Effects of sodium restriction and hydrochlorothiazide on RAAS blockade efficacy in diabetic nephropathy: A randomised clinical trial. *Lancet Diabetes Endocrinol* 2: 385-395, 2014

11. Vegter S, Perna A, Postma MJ, Navis G, Remuzzi G, Ruggenenti P: Sodium intake, ACE inhibition, and progression to ESRD. *J Am Soc Nephrol* 23: 165-173, 2012

12. Lambers Heerspink HJ, Holtkamp FA, Parving HH, Navis GJ, Lewis JB, Ritz E, de Graeff PA, de Zeeuw D: Moderation of dietary sodium potentiates the renal and cardiovascular protective effects of angiotensin receptor blockers. *Kidney Int* 82: 330-337, 2012

13. Mizobuchi M, Morrissey J, Finch JL, Martin DR, Liapis H, Akizawa T, Slatopolsky E: Combination therapy with an angiotensin-converting enzyme inhibitor and a vitamin D analog suppresses the progression of renal insufficiency in uremic rats. *J Am Soc Nephrol* 18: 1796-1806, 2007

14. Zhang Z, Zhang Y, Ning G, Deb DK, Kong J, Li YC: Combination therapy with AT1 blocker and vitamin D analog markedly ameliorates diabetic nephropathy: Blockade of compensatory renin increase. *Proc Natl Acad Sci U S A* 105: 15896-15901, 2008

15. de Borst MH, Hajhosseiny R, Tamez H, Wenger J, Thadhani R, Goldsmith DJ: Active vitamin D treatment for reduction of residual proteinuria: A systematic review. *J Am Soc Nephrol* 24: 1863-1871, 2013

16. de Zeeuw D, Agarwal R, Amdahl M, Audhya P, Coyne D, Garimella T, Parving HH, Pritchett Y, Remuzzi G, Ritz E, Andress D: Selective vitamin D receptor activation with paricalcitol for reduction of albuminuria in patients with type 2 diabetes (VITAL study): A randomised controlled trial. *Lancet* 376: 1543-1551, 2010

17. Li YC, Kong J, Wei M, Chen ZF, Liu SQ, Cao LP: 1,25-dihydroxyvitamin D(3) is a negative endocrine regulator of the renin-angiotensin system. *J Clin Invest* 110: 229-238, 2002

18. Vaidya A, Sun B, Larson C, Forman JP, Williams JS: Vitamin D3 therapy corrects the tissue sensitivity to angiotensin ii akin to the action of a converting enzyme inhibitor in obese hypertensives: An interventional study. *J Clin Endocrinol Metab* 97: 2456-2465, 2012

19. Mirkovic K, Frenay AS, van den Born J, van Goor H, Navis G, de Borst MH, NIGRAM consortium: Sodium restriction potentiates the renoprotective effects of combined vitamin D receptor activation and angiotensin-converting enzyme inhibition in established proteinuric nephropathy. *Nephrol Dial Transplant,* doi: 10.1093/ndt/gfv304, 2015

20. Keyzer CA, Lambers-Heerspink HJ, Joosten MM, Deetman PE, Gansevoort RT, Navis G, Kema IP, de Zeeuw D, Bakker SJ, de Borst MH, PREVEND Study Group: Plasma vitamin D level and change in albuminuria and eGFR according to sodium intake. *Clin J Am Soc Nephrol* 10: 2119-2127, 2015

21. De Nicola L, Conte G, Russo D, Gorini A, Minutolo R: Antiproteinuric effect of add-on paricalcitol in CKD patients under maximal tolerated inhibition of renin-angiotensin system: A prospective observational study. *BMC Nephrol* 13: 150-2369-13-150, 2012

22. Agarwal R, Acharya M, Tian J, Hippensteel RL, Melnick JZ, Qiu P, Williams L, Batlle D: Antiproteinuric effect of oral paricalcitol in chronic kidney disease. *Kidney Int* 68: 2823-2828, 2005

23. Alborzi P, Patel NA, Peterson C, Bills JE, Bekele DM, Bunaye Z, Light RP, Agarwal R: Paricalcitol reduces albuminuria and inflammation in chronic kidney disease: A randomized double-blind pilot trial. *Hypertension* 52: 249-255, 2008

24. Fishbane S, Chittineni H, Packman M, Dutka P, Ali N, Durie N: Oral paricalcitol in the treatment of patients with CKD and proteinuria: A randomized trial. *Am J Kidney Dis* 54: 647-652, 2009

25. Larsen T, Mose FH, Bech JN, Pedersen EB: Effect of paricalcitol on renin and albuminuria in non-diabetic stage III-IV chronic kidney disease: A randomized placebo-controlled trial. *BMC Nephrol* 14: 163-2369-14-163, 2013

26. Hojs N, Bevc S, Balon BP, Hojs R, Ekart R: Paricalcitol reduces proteinuria in non-dialysis chronic kidney disease patients. *Ther Apher Dial* 17: 368-372, 2013

27. Heeg JE, de Jong PE, van der Hem GK, de Zeeuw D: Efficacy and variability of the antiproteinuric effect of ACE inhibition by lisinopril. *Kidney Int* 36: 272-279, 1989

28. Fabris B, Jackson B, Johnston CI: Salt blocks the renal benefits of ramipril in diabetic hypertensive rats. *Hypertension* 17: 497-503, 1991

29. Ying WZ, & Sanders PW: Dietary salt modulates renal production of transforming growth factor-beta in rats. *Am J Physiol* 274: F635-41, 1998

30. Kwakernaak AJ, Waanders F, Slagman MC, Dokter MM, Laverman GD, de Boer RA, Navis G: Sodium restriction on top of renin-angiotensin-aldosterone system blockade increases circulating levels of N-acetyl-seryl-aspartyl-lysyl-proline in chronic kidney disease patients. *J Hypertens* 31: 2425-2432, 2013

31. Slagman MC, Nguyen TQ, Waanders F, Vogt L, Hemmelder MH, Laverman GD, Goldschmeding R, Navis G: Effects of antiproteinuric intervention on elevated connective tissue growth factor (CTGF/CCN-2) plasma and urine levels in nondiabetic nephropathy. *Clin J Am Soc Nephrol* 6: 1845-1850, 2011

32. de Borst MH, & Navis G: Sodium intake, RAAS-blockade and progressive renal disease. *Pharmacol Res* 107: 344-351, 2016

33. Kuhlmann A, Haas CS, Gross ML, Reulbach U, Holzinger M, Schwarz U, Ritz E, Amann K: 1,25-dihydroxyvitamin D3 decreases podocyte loss and podocyte hypertrophy in the subtotally nephrectomized rat. *Am J Physiol Renal Physiol* 286: F526-33, 2004

34. Zhang Y, Kong J, Deb DK, Chang A, Li YC: Vitamin D receptor attenuates renal fibrosis by suppressing the renin-angiotensin system. *J Am Soc Nephrol* 21: 966-973, 2010

35. Yuan W, Pan W, Kong J, Zheng W, Szeto FL, Wong KE, Cohen R, Klopot A, Zhang Z, Li YC: 1,25-dihydroxyvitamin D3 suppresses renin gene transcription by blocking the activity of the cyclic AMP response element in the renin gene promoter. *J Biol Chem* 282: 29821-29830, 2007

36. He W, Kang YS, Dai C, Liu Y: Blockade of Wnt/beta-catenin signaling by paricalcitol ameliorates proteinuria and kidney injury. *J Am Soc Nephrol* 22: 90-103, 2011

37. Schwarz U, Amann K, Orth SR, Simonaviciene A, Wessels S, Ritz E: Effect of 1,25 (OH)2 vitamin D3 on glomerulosclerosis in subtotally nephrectomized rats. *Kidney Int* 53: 1696-1705, 1998

38. de Boer IH, Sachs M, Hoofnagle AN, Utzschneider KM, Kahn SE, Kestenbaum B, Himmelfarb J: Paricalcitol does not improve glucose metabolism in patients with stage 3-4 chronic kidney disease. *Kidney Int* 83: 323-330, 2013

39. Lundwall K, Jorneskog G, Jacobson SH, Spaak J: Paricalcitol, microvascular and endothelial function in non-diabetic chronic kidney disease: A randomized trial. *Am J Nephrol* 42: 265-273, 2015

40. Beveridge LA, Struthers AD, Khan F, Jorde R, Scragg R, Macdonald HM, Alvarez JA, Boxer RS, Dalbeni A, Gepner AD, Isbel NM, Larsen T, Nagpal J, Petchey WG, Stricker H, Strobel F, Tangpricha V, Toxqui L, Vaquero MP, Wamberg L, Zittermann A, Witham MD, D-PRESSURE Collaboration: Effect of vitamin D supplementation on blood pressure: A systematic review and meta-analysis incorporating individual patient data. *JAMA Intern Med* 175: 745-754, 2015

41. Apperloo AJ, de Zeeuw D, de Jong PE: A short-term antihypertensive treatment-induced fall in glomerular filtration rate predicts long-term stability of renal function. *Kidney Int* 51: 793-797, 1997

42. Holtkamp FA, de Zeeuw D, Thomas MC, Cooper ME, de Graeff PA, Hillege HJ, Parving HH, Brenner BM, Shahinfar S, Lambers Heerspink HJ: An acute fall in estimated glomerular filtration rate during treatment with losartan predicts a slower decrease in long-term renal function. *Kidney Int* 80: 282-287, 2011

43. Agarwal R, Hynson JE, Hecht TJ, Light RP, Sinha AD: Short-term vitamin D receptor activation increases serum creatinine due to increased production with no effect on the glomerular filtration rate. *Kidney Int* 80: 1073-1079, 2011

44. Scialla JJ, & Wolf M: Roles of phosphate and fibroblast growth factor 23 in cardiovascular disease. *Nat Rev Nephrol* 10: 268-278, 2014

45. Keyzer CA, de Jong MA, Fenna van Breda G, Vervloet MG, Laverman GD, Hemmelder M, Janssen WM, Lambers Heerspink HJ, Navis G, de Borst MH, Holland Nephrology Study (HONEST) Network: Vitamin D receptor activator and dietary sodium restriction to reduce residual urinary albumin excretion in chronic kidney disease (ViRTUE study): Rationale and study protocol. *Nephrol Dial Transplant* 31: 1081-1087, 2016

46. Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF,3rd, Feldman HI, Kusek JW, Eggers P, Van Lente F, Greene T, Coresh J, CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration): A new equation to estimate glomerular filtration rate. *Ann Intern Med* 150: 604-612, 2009

Supplemental Table 1. Baseline characteristics per treatment sequence

	Treatment sequence*			
Characteristics	**A (n=9)**	**B (n=12)**	**C (n=12)**	**D (n=12)**
Age, years	54.5 ± 15.8	53.2 ± 16.3	52.6 ± 10.7	46.9 ± 10.4
Male sex, n (%)	7 (78)	8 (67)	10 (83)	8 (67)
White ethnicity, n (%)	9 (100)	12 (100)	12 (100)	12 (100)
Body mass index, kg/m^2	29.2 ± 4.8	26.6 ± 3.2	28.2 ± 4.5	28.5 ± 5.9
Renal diagnosis				
IgA nephropathy, n (%)	1 (11)	4 (33)	2 (17)	2 (17)
Focal segmental glomerulosclerosis, n (%)	3 (33)	2 (17)	1 (8)	2 (17)
Membranous nephropathy, n (%)	2 (22)	2 (17)	1 (8)	3 (25)
Hypertensive nephropathy, n (%)	1 (11)	0 (0)	2 (17)	1 (8)
Other/Inconclusive, n (%)	2 (22)	4 (33)	6 (50)	4 (33)
Season				
Winter, n (%)	1 (11)	3 (25)	4 (33)	3 (25)
Spring, n (%)	2 (22)	4 (33)	3 (25)	1 (8)
Summer, n (%)	3 (33)	4 (33)	4 (33)	8 (67)
Autumn, n (%)	3 (33)	1 (8)	1 (8)	0 (0)
Medication use†				
ACE inhibitor, n (%)	9 (100)	12 (100)	12 (100)	12 (100)
β-blocker, n (%)	2 (22)	2 (17)	4 (33)	4 (33)
Calcium channel blocker, n (%)	1 (11)	5 (42)	5 (42)	3 (25)
α-blocker, n (%)	1 (11)	0 (0)	1 (8)	1 (8)
Diuretic, n (%)	4 (44)	2 (17)	2 (17)	4 (33)
Lipid lowering agent, n (%)	6 (67)	6 (50)	7 (58)	5 (42)
Systolic blood pressure, mmHg	126 ± 15	125 ± 9	125 ± 11	126 ± 9

Characteristics	Treatment sequence* A (n=9)	B (n=12)	C (n=12)	D (n=12)
Diastolic blood pressure, mmHg	77 ± 10	76 ± 6	77 ± 7	76 ± 9
eGFR (CKD-EPI), ml/min/1.73m2	73 ± 17	71 ± 30	65 ± 23	70 ± 23
Calcium, mmol/L	2.36 ± 0.12	2.35 ± 0.11	2.36 ± 0.09	2.36 ± 0.11
Phosphate, mmol/L‡	0.86 ± 0.17	0.94 ± 0.14	0.87 ± 0.20	1.07 ± 0.11
Albuminuria, mg/24h	1,372 [822-2,290]	1,154 [654-2,037]	1,295 [783-2,143]	951 [561-1,612]
Urinary protein excretion, g/24h	1.59 [0.96-2.65]	1.45 [0.88-2.39]	1.56 [0.99-2.44]	1.19 [0.77-1.83]
Urinary sodium excretion, mmol/24h	173 ± 56	192 ± 25	160 ± 45	175 ± 104
Creatinine clearance, mL/min	106 ± 24	99 ± 43	99 ± 36	100 ± 47

* A= placebo-regular sodium diet, paricalcitol-regular sodium diet, placebo-sodium restriction diet, paricalcitol-sodium restriction diet.; B= paricalcitol-regular sodium diet, placebo-regular sodium diet, paricalcitol-sodium restriction diet, placebo- sodium restriction diet; C= placebo-sodium restriction diet, paricalcitol-sodium restriction diet, placebo-regular sodium diet, paricalcitol-regular sodium diet; D= paricalcitol-sodium restriction diet, placebo-sodium restriction diet, paricalcitol-regular sodium diet, placebo-regular sodium diet.
Data are presented as mean ± SD, geometric mean [95% CI], and number (percentage) for normally, skewed distributed data, and nominal data, respectively. Differences between the four sequences were assessed with ANOVA for normally distributed continuous data, the Kruskal-Wallis test for skewed distributed data, and the $\chi 2$ test for nominal data. ‡ $P<0.05$. † At the end of the run-in period, all patients were treated with ramipril 10 mg once daily, except for one patient who received ramipril 5 mg due to low blood pressure. In one patient, diuretic therapy was stopped during the study and later on resumed because of oedema. In another patient the calcium channel blocker was stopped due to symptomatic hypotension. All other non-study-related medication was kept stable during the study periods.

Supplemental Table 2. Adverse effects possibly or probably related to treatment

	Regular sodium diet		Sodium restriction diet	
	Placebo N = 44	Paricalcitol N= 44	Placebo N = 43	Paricalcitol N = 43
Laboratory				
Hypercalcemia *corrected calcium > 2.60 mmol/L*	4	3	2	8
Hypoparathyroidism PTH < 1.5 pmol/L	0	2	0	2
Elevated liver enzymes ASAT > 40 U/L, ALAT > 45 U/L, GGT > 50 U/L	9	14	8	7
Anaemia	0	0	1	1
Acute-on-chronic kidney disease	0	0	0	1
Hyperkalaemia *potassium > 5.0 mmol/L*	2	3	4	5
Hypokalaemia *potassium < 3.50 mmol/L*	0	2	0	0
Hyponatraemia *sodium < 135 mmol/L*	1	0	1	0
Hypophosphataemia *phosphate <0.80 mmol/L*	10	5	6	2
Hypocalcaemia *corrected calcium <2.20 mmol/L*	2	2	3	1
Elevated alkaline phosphatase *ALP > 150 U/L*	0	1	2	0
Worsening hypothyroidism	0	1	0	0
Rhabdomyolysis	0	1	0	0
Physical				
Peripheral oedema	13	12	8	5
De novo atrial fibrillation	0	0	1	0
Foot drop	0	0	1	0
Peripheral artery occlusive disease (Fontaine IIB)	1	0	0	0
Reported adverse effects				
Severe symptomatic hypotension	0	0	0	1
Mild symptomatic hypotension	2	1	10	4
Fatigue	1	5	3	7
Malaise	2	2	1	2
Headache	7	7	4	3
Vertigo	2	4	4	3
Visual complaint	1	2	0	1
Dry mouth	1	1	2	1
Itchiness	1	1	1	0

	Regular sodium diet		Sodium restriction diet	
	Placebo	Paricalcitol	Placebo	Paricalcitol
	N = 44	N= 44	N = 43	N = 43
Skin complaint	2	0	0	1
Excessive sweating	0	1	0	0
Dyspnoea	1	2	1	0
Dry cough	3	4	2	1
Lower respiratory tract infection	1	0	1	1
Palpitations	0	0	2	1
Gastrointestinal complaints*	1	2	7	3
Pain	5	5	4	3
Myalgia	5	5	4	6
Muscle spasm or cramp†	3	0	1	2
Arthritis including gout	3	3	3	2
Bursitis	0	1	1	1
Micturition complaints including urinary tract infection	2	4	1	0
Erectile dysfunction†	1	0	0	0

Data represent numbers of patients with a particular adverse effect per study period. Some patients had more than one treatment-related adverse effect. * Gastrointestinal complaints including heartburn, dyspepsia, constipation and diarrhoea. † Two patients had complaints possibly related to ramipril (muscle pain and erectile dysfunction) and switched to another ACEi (enalapril 40 mg/day and fosinopril 10 mg/day, resp.).

Supplemental Table 3. Clinical parameters during four treatment periods; *per protocol* analysis

	Regular sodium diet		Sodium restriction diet	
	Placebo	Paricalcitol	Placebo	Paricalcitol
	N= 31	N= 34	N= 34	N= 32
Plasma/Serum				
Hb, mmol/L	9.1 [8.8-9.4]	9.1 [8.9-9.4]	9.1 [8.8-9.4]	9.1 [8.8-9.4]
Sodium, mmol/L	140 [139-141]	140 [139-141]	140 [139-140]	140 [139-141]
Potassium, mmol/L	4.3 [2.7-5.9]	4.2 [2.5-5.9]	4.4 [2.6-6.1]†	4.4 [3.1-5.6]†
Calcium, mmol/L	2.35 [2.09-2.60]	2.37 [2.13-2.61]	2.36 [2.14-2.59]	2.41 [1.75-3.07]*
Phosphate, mmol/L	0.91 [0.85-0.98]	0.95 [0.90-1.01]*	0.93 [0.88-0.96]	0.97 [0.91-1.03]*
Creatinine, µmol/L	112 [85-140]	114 [86-141]	112 [85-139]	121 [93-149]*†‡
eGFR, ml/min/1.73m^2	68 [59-76]	66 [58-74]	67 [59-75]	62 [53-70]*†‡
Albumin, g/L	39 [37-41]	39 [38-41]	40 [38-41]	40 [38-41]*
Total cholesterol, mmol/L	5.1 [4.7-5.5]	5.1 [4.7-5.6]	4.7 [4.3-5.1]*†	4.9 [4.5-5.4]†‡
HDL cholesterol, mmol/L	1.4 [1.2-1.5]	1.3 [1.2-1.5]	1.3 [1.1-1.4]*†	1.3 [1.2-1.4]
LDL cholesterol, mmol/L	3.0 [2.7-3.3]	3.0 [2.7-3.4]	2.8 [2.5-3.1]	2.9 [2.6-3.3]
Renin, pg/mL	44.0 [17.1-113.1]	48.6 [18.8-125.5]	60.3 [23.4-155.7]*†	62.7 [24.2-162.4]*†
PTH, pmol/L	5.3 [4.5-6.2]	3.6 [3.1-4.3]*	5.4 [4.7-6.3]†	3.4 [2.8-4.0]*‡
25(OH)D, nmol/L	53.5 [45.1-61.8]	52.2 [44.1-60.3]	52.6 [44.8-60.3]	58.0 [49.4-66.6]
FGF23, RU/mL	115 [100-132]	141 [120-166]*	123 [106-142]†	156 [130-188]*†‡
Urine				
Creatinine, mmol/24h	15.5 [14.2-16.9]	15.0 [13.8-16.3]	14.6 [13.3-15.8]	15.2 [14.1-16.4]
Sodium, mmol/24h	187 [164-210]	183 [163-202]	105 [88-123]*†	112 [96-129]*†
Urea, mmol/24h	446 [402-490]	427 [383-471]	401 [359-443]*	422 [382-462]
Potassium, mmol/24h	85 [76-94]	84 [75-93]	86 [76-97]	88 [77-98]
Calcium, mmol/24h	3.2 [2.5-3.8]	5.1 [4.1-6.1]*	2.6 [1.8-3.4]†	4.5 [3.6-5.5]*‡
Phosphate, mmol/24h	33.8 [30.6-36.9]	33.9 [29.9-37.9]	32.6 [27.4-37.9]	32.0 [28.8-35.2]
Albuminuria, mg/24h	1,177 [823-1,682]	1,082 [772-1,516]	804 [564-1,146]*†	690 [480-993]*†‡
Proteinuria, g/24h	1.5 [1.1-2.1]	1.4 [1.0-1.9]	1.1 [0.8-1.5]*†	1.0 [0.7-1.3]*†
Albumin/creatinine ratio	77 [54-109]	74 [52-103]	57 [40-81]*†	46 [32-67]*†‡
Creatinine clearance, mL/min	105 [88-121]	98 [85-111]	97 [83-110]	93 [81-104]*
Other				
Systolic blood pressure, mmHg	129 [125-134]	125 [120-129]*	120 [116-125]*†	121 [116-125]*
Diastolic blood pressure, mmHg	79 [76-82]	77 [73-81]	74 [70-77]*†	74 [70-77]*
Mean arterial pressure, mmHg	96 [92-99]	93 [89-97]*	89 [86-93]*†	90 [86-93]*
Heart rate, bpm	65 [61-69]	66 [62-70]	66 [62-70]	64 [60-68]†‡
Body weight, kg	95 [89-101]	95 [89-100]	93 [87-98]*†	93 [87-98]*†

Data are presented as estimated mean [95% CI] or estimated geometric mean [95% CI] for normally or skewed distributed data, respectively. P value shows treatment effect by linear mixed modelling with centre, treatment, sequence and the interaction treatment*sequence as fixed factors.

*P< 0.05 versus placebo on regular sodium diet

†P<0.05 versus paricalcitol on regular sodium diet

‡P<0.05 versus placebo on sodium restriction diet

7

Chapter 8

SUMMARY AND FUTURE PERSPECTIVES

Summary

Patients with chronic kidney disease (CKD) are characterized by an increased risk, not only for progression of CKD, but even more so for cardiovascular events. The main goal in treatment of patients with CKD is to improve outcome, which therefore not only requires protection from CKD progression, but importantly also prevention of cardiovascular complications. Currently, the strategy for cardiovascular risk management is to treat traditional risk factors (blood pressure, obesity, smoking, dyslipidemia and diabetes if applicable) and CKD specific risk factors (proteinuria, anemia, vitamin D deficiency and possibly elevated FGF23) in isolation. Unfortunately, despite optimizing risk profiles, the protection against CVD remains far from satisfying. In part, this may be explained by interactions between risk factors, by residual gaps in our understanding of the pathophysiology of CVD in CKD and untoward effects of pharmacological treatment. This is exemplified by the finding that active vitamin D treatment, while possibly having beneficial effects on proteinuria as discussed below, may raise FGF23, which in turn may counterbalance the presumed beneficial effect of proteinuria reduction.

This thesis focused specifically on the interaction between different CKD specific risk factors for CVD in patients with kidney failure. This concept is illustrated in figure 1 in **chapter 1**. FGF23, iron deficiency, vitamin D deficiency and proteinuria have been introduced as important risk factors for CVD and the direct effects of these different risk factors on cardiovascular risk are described. Currently, the knowledge about connections between these risk factors is expanding and the newly discovered regulating pathways are described. However, there are still important pieces of the puzzle missing in this highly complex mechanism, which triggered our curiosity and in order to clarify the gap in our knowledge, we have carried out the studies described in this thesis (figure1)

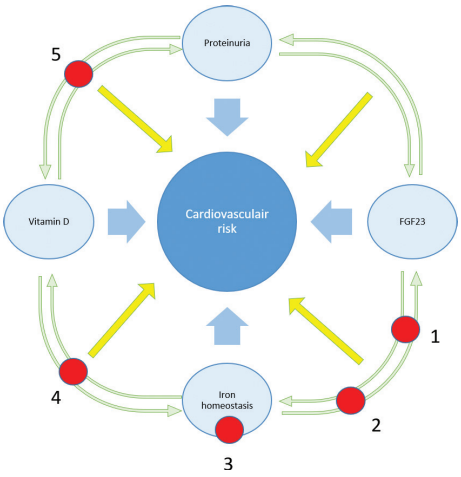


Figure 1. The isolated impact of CKD specific risk factors like FGF23, proteinuria, vitamin D deficiency and iron deficiency on CVD has been investigated last decade (yellow arrows). During last years, more evidence has emerged pointing to amplifying effects among these risk factors (green arrows). This thesis focused on the interaction among different CKD specific risk factors for CVD in patients with kidney failure (red bullets). Explanations for the numbers follow in the text below.

In **chapter 2**, we focused on the association between FGF23 and red cell distribution width (RDW) as two major risk factors for CVD (1-3). RDW is a measure of the variability in size of circulating erythrocytes and is strongly associated with increased risk for cardiovascular disease (4-6). Iron deficiency is a clinical condition in which RDW is elevated, caused by ineffective red blood cell production and increased retrieval from the circulation (7). It is known that iron deficiency also induces expression of FGF23 followed by increased cleavage of biologically active intact FGF23 (iFGF23) into inactive fragments including c-terminal FGF23 (cFGF23), leaving circulating iFGF23 levels unchanged as a net result (8). Like RDW, FGF23 is associated with cardiovascular risk but currently it is not clear whether and how the ratio of iFGF23 and cFGF23 contributes to these poor outcomes. In order to elucidate the relative contribution of these factors to the risk profile of patients with CKD, we hypothesized that a higher RDW is associated with more FGF23 cleavage, providing a common pathway in which both markers lead to adverse outcome *(figure 1, bullet 1).* We performed a post-hoc analysis of baseline data from a cohort of 52 patients with CKD and chronic heart failure (CHF) enrolled in the EPOCARES trial and examined the relationship between cFGF23, iFGF23 and RDW as a marker of iron deficiency. Our analysis showed a statistically significant positive relation between cFGF23 and RDW ($\beta = 1.63 \times 10^{-3}$, $P < 0.001$), but not between iFGF23 and RDW ($\beta = 1.38 \times 10^{-3}$, $P = 0.336$). After correction for parameters of renal function, phosphate metabolism and inflammation, the association between cFGF23 and RDW persisted. Remarkably, correction for iron status (TSAT and ferritin) did also have no effect on the relationship between cFGF23 and RDW, suggesting that other underlying mechanisms explain the link between these two risk factors.

In **chapter 3**, we investigated the role of different iron conditions on FGF23 metabolism in healthy mice and mice after 5/6 nephrectomy *(figure 1, bullet 2)*. Since it has been described that ferric carboxymaltose (FCM), but not iron dextran (ID), can induce hypophosphatemia, several studies have been undertaken to unravel the mechanism underlying this feature. FGF23 may play a role in this iron-induced hypophosphatemia and therefore we investigated the effect of treatment with either FCM or ID on iFGF23 and cFGF23 levels, besides phosphate excretion, in our experimental groups. Despite the clear differences in ferritin levels and creatinine levels, supporting the validity of the experimental mouse models, no differences were observed for cFGF23 and iFGF23 levels between different iron groups. Additionally, FCM and ID didn't induce different patterns of iFGF23 and cFGF23 levels. In order to test the role of iron status on FGF23 sensitivity, recombinant FGF23 was given during the last 24 hours of the experiments. No effect on fractional phosphate excretion was observed among the study groups. Because of these remarkable and unexpected results, especially the lack of effect of low-iron conditions, we reevaluated the difference in iron status in our model. Additional measurement of iron concentrations in mice livers showed no differences in iron content between mice with iron deficiency and iron loading and this could indicate that ferritin concentrations may be a poor reflection of iron status in a relatively short-term mouse model of iron deficiency. To answer the original hypothesis and unravel the elusive metabolic connection between iron and FGF23 concentrations and bioactivity, we are currently planning to perform additional experiments in mice with more pronounced differences in iron status.

Renal anemia is a common complication in CKD patients and is consistently associated with cardiovascular risks (9-12). Hepcidin is the main regulatory protein of systemic iron metabolism and contributes to development of renal anemia in CKD patients due to internalization of the iron transporter ferroportin in enterocytes, macrophages and hepatocytes leading to functional iron deficiency (13). Hepcidin is primarily expressed in the liver and is down regulated in response to low iron stores, anemia and hypoxia, thereby facilitating iron uptake and bioavailability (14). Conversely, hepcidin expression is upregulated by iron overload and inflammation. Due to micro-inflammation in a uremic environment, hepcidin expression in CKD patients is increased leading to functional iron deficiency contributing subsequently to renal anemia. Most studies focused on the function and regulation of hepcidin in the liver as the main hepcidin production site but accumulating data suggest production of hepcidin in other tissues as well, like the myocardium (15, 16). Haddad et al. showed that iron deficiency in cardiomyocytes impaired mitochondrial respiration and hampered adaptation to acute and chronic increase in workload (17). FCM supplementation restored cardiac energy reserve and function in iron-deficient hearts. In patients with heart failure and iron deficiency, iron administration improved symptoms and exercise and may reduce the number of hospital admissions, independent of its effect on anemia (18, 19). However, the role

of hepcidin expression in the heart has not been clarified yet, while it can be speculated that local expression might modulate iron availability to cardiomyocytes. In vitro, hepcidin is thought to act in an autocrine fashion to regulate cardiac iron turnover (20). To get insight in hepcidin expression at the tissue level, we investigated the hypothesis that hepcidin expression in liver and heart is differentially regulated *(figure 1, bullet 3)*. In **chapter 4**, we hypothesized that cardiac hepcidin expression is upregulated in response to damage and is related to the severity of cardiac injury and increased local iron content. We studied the expression of hepcidin in liver and cardiac tissue in response to myocardial infarction and/or CKD. To this end, rats were subjected to subtotal nephrectomy and/or coronary ligation or sham surgery to create 4 groups: control, rats with CKD, rats with myocardial infarction and rats with both CKD and myocardial infarction. Cardiac hepcidin mRNA expression was increased in rats with myocardial infarction and even more in CKD rats. Rats with both myocardial infarction and CKD showed the highest increase in cardiac hepcidin mRNA expression. Remarkably, cardiac ferritin staining was not different among groups. However, cardiac hepcidin mRNA expression correlated significantly with injury markers of the heart (BNP and CTGF). In contrast, liver hepcidin expression was unaffected by myocardial infarction or CKD, while it was significantly decreased in rats with both myocardial infarction and CKD. This study indicates that hepcidin regulation is different in liver and heart and suggests a role for cardiac injury rather than (local) iron status as inducer for cardiac hepcidin expression in cardiorenal failure.

Besides iron metabolism and epo deficiency, vitamin D plays a role in renal anemia. In **chapter 5**, we reviewed the literature to provide an overview of the potential link between the vitamin D system and erythropoiesis *(figure 1, bullet 4)*. Epidemiological studies show a clear association between vitamin D deficiency and anemia (21, 22) but the pathophysiological mechanism behind this association has not been fully explained. Several hypotheses are formulated to explain a potential causal connection. We concluded that high dose vitamin D therapy suppresses hepcidin expression directly but also indirectly by reducing inflammatory cytokines that induce hepcidin. This would improve bioavailability of iron for erythropoiesis. We also noted that there is evidence for a direct positive effect of vitamin D on erythroid precursors with a synergistic action when combined with epoetin. The effect of vitamin D on HIF-1α is described and the assumed impact of vitamin D on inflammatory cytokines leading to anemia of chronic disease is discussed. Thereafter, the interaction of the vitamin D receptor (VDR) and the EPO receptor is discussed and in addition several hypotheses about the role of dysregulation of the parathyroid gland leading to hyperparathyroidism in developing renal anemia are described.

Another major risk factor contributing to CKD progression and dismal cardiovascular outcome is albuminuria (23). Despite optimal treatment with pharmacological blockade of the renin-

angiotensin-aldosterone system (RAAS) by angiotensin converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARB), residual albuminuria exists in many CKD patients, which is associated with unfavorable renal and cardiovascular outcomes (24, 25). In addition to RAAS blockade, both active vitamin D treatment and dietary sodium restriction are known to possess additional antiproteinuric effects (26-28). However, whether the capacity of active vitamin D to lower residual albuminuria depends on dietary sodium intake has been unresolved. We therefore designed an interventional trial (**chapter 6**) to prospectively investigate the separate and combined effect on albuminuria of paricalcitol and dietary sodium restriction in non-diabetic CKD patients treated with a fixed dose of RAAS blockade *(figure 1, bullet 5).* In **chapter 7**, we conclude that the combination of paricalcitol with dietary sodium restriction provided the strongest reduction of residual albuminuria during fixed dose ACEi therapy. However, patients with dietary sodium restriction showed no additional significant albuminuria reduction when co-treated with paricalcitol compared with patients with dietary sodium restriction and placebo. Furthermore, absolute reduction of albuminuria was highest in low versus high sodium restriction instead of paricalcitol versus placebo. Based on these findings, we conclude that reduction of albuminuria was mainly driven by restricting dietary sodium intake.

General discussion

For many years, attempts have been undertaken to adequately protect CKD patients from CVD. Modifiable risk factors specific for CKD have been identified, like anemia, iron deficiency, vitamin D deficiency and proteinuria. Research has been performed in order to study the effect of modifying these risk factors, either pharmacologically or by diet. Recently it has been recognized that these risk factors not only influence clinical outcomes, but also interact with each other. These insights may lead to further optimizing risk management.

In this thesis we postulate the concept of intertwined relationships between CKD specific risk factors. We suggest that intervening in one risk factor might have adverse (or beneficial) effects on the other risk factor. We argue for a more integrated concept and we believe that research should be done taking into account the whole cascade of risk factors. As an example, we highlight the ongoing discussion about potential beneficial effects of iron therapy in CKD, which is not fully settled as exemplified by the contradictory results of the FIND-CKD and REVOKE trials (29, 30). The multicenter, multinational FIND-CKD study of 626 patients with non-dialysis dependent CKD showed that intravenous iron treatment targeting high ferritin levels (400-600 µg/L) improved anemia management, with no safety concerns in terms of CV events. Conversely, in the single-center REVOKE trial, 136 patients with CKD and iron deficient anemia were randomly assigned

to either oral or intravenous iron therapy in order to compare the effect on progression of CKD. This trial was terminated early based on little chance of finding differences in measured GFR slopes at interim analyses, but a higher incidence rate of serious adverse events in the intravenous iron treatment group, including CV complications. In addition to data from RCT's, a number of epidemiological studies pertaining intravenous iron safety and efficacy have been published with contradictory results. An analysis of data from the Dialysis Outcomes and Practice Patterns study (DOPPS) considering 32435 patients showed an association between high intravenous iron and mortality (31). In contrast, an analysis from the Developing Evidence to Inform Decisions about Effectiveness (DEcIDE)-ESRD study in 14078 HD patients showed no associations of intravenous iron with mortality (32). More rigorous scientific evaluation of the use of high dose iron therapy in patients with kidney failure was needed and encouraged the researchers of the recently published PIVOTAL trial to conduct a non-inferiority trial on safety and efficacy of a high-dose regimen of intravenous iron administered proactively (400 mg monthly, unless the ferritin concentration was >700 µg/L or the transferrin saturation was > 40%) compared with low dose intravenous iron reactively (0 to 400 mg monthly, with a ferritin concentration of <200 µg/L or a transferrin saturation of <20% being a trigger for iron administration) in 2141 hemodialysis patients (33). Among hemodialysis patients, the high dose intravenous iron regimen administered proactively was superior to a low dose regimen administered reactively. Although the differences were small in absolute terms, the use of a high-dose regimen of intravenous iron proactively resulted in significantly lower risk of death or major nonfatal cardiovascular events and lower ESA use as compared with patients who received a reactive, low-dose iron regimen. Incidence of infection and hospitalization for any cause did not differ significantly. Reconciling the conclusions of these studies, one could speculate that some individuals may benefit from iron administration, while others do not, possibly because of currently undetermined causes. In our concept of intertwined relationships between risk factors, it would be plausible to measure both iFGF23 and cFGF23 levels in addition to measuring iron markers. An unfavorable shift in the iFGF23/cFGF23 ratio could explain the variable effect of iron on cardiovascular risk. This may provide an answer to the question whether the diverging effects of iron on cardiovascular risk may be mediated by individually determined effects on FGF23 metabolism in patients with CKD. However, if this theory is correct, the causes of diverging effects of iron on FGF23 metabolism between individual patients, require further research.

In the search for the role of iron on FGF23 metabolism, Farrow et al demonstrated that iron deficiency stimulates FGF23 transcription which is counterbalanced by an increased cleavage of iFGF23 into cFGF23 within the osteocytes to prevent release on non-physiologically high bioactive FGF23, which would induce hypophosphatemia (34). Similarly, in female patients with

iron deficient anemia markedly elevated C-terminal FGF23 (cFGF23) levels but not intact FGF23 (iFGF23) levels were found (35). In accordance with these results, we found a robust association between cFGF23 and RDW but not between iFGF23 and RDW in patients with both CHF and CKD. As mentioned in chapter 2, both cFGF23 and RDW are associated with adverse clinical outcomes in patients with CKD (36-38). In order to determine the effect of iron on CVD in CKD, it is necessary to understand whether this effect is mediated by RDW and/ or cFGF23, because this knowledge would change therapeutic decisions on an individual basis. We speculate that patients who benefit from iron therapy are the ones in which this intervention induces a decrease in cFGF23 and/or RDW. Since studies reported some evidence for an association between cFGF23 and dismal outcomes (37, 39), that would imply that patients that do not benefit from iron therapy, might be characterized by a lack of reduction of cFGF23 and/or RDW. By gaining insight into collateral effects of iron on cFGF23 and RDW, it could be possible to delineate a more differentiated risk profile and follow the effects of its management for an individual patient in order to be able to estimate whether treating with IV iron is beneficial or not.

This concept of amplifying or attenuating the impact of risk factors seems even more complex due to our finding that the regulation of hepcidin is regulated differently in liver and other tissues, in particular the heart. This finding provides a new perspective because the effects of hepcidin (and perhaps also other risk factors) may not be accurately reflected by its serum concentrations, but by its local tissue expression instead. Since the results of the FAIR-HF trial were published (18), it is generally accepted that treatment with intravenous iron therapy in patients with chronic heart failure and iron deficiency, with or without anemia, improves cardiac function. Recently, this conclusion was substantiated in both animal (40) and human isolated cardiomyocytes (41). Since we demonstrated that hepcidin regulation in the heart can be different from that in the liver and taking into account that cardiomyocytes benefit from normal iron status, it is conceivable that cardiac hepcidin functions to counteract the effects of reduced systemic iron availability by promoting iron retention within the cardiomyocyte. However, instead of hepcidin expression in the liver, our data suggest a role for injury rather than iron status as an inducer for cardiac expression of hepcidin. This new understanding raises questions on the interplay between systemic and local iron control in the context of heart failure and CKD. Having insight into possible interactions at tissue levels beside interactions on systemic levels might make it possible to fine-tune therapies targeting the systemic or local hepcidin/ferroportin axis. Unfortunately, clinically quantifying molecular patterns at the tissue level is currently not possible.

In this thesis, we demonstrate that the mainstay of proteinuria reduction, on top of adequate RAS inhibition, with limited additional effect of paricalcitol. Furthermore, we demonstrated that the

additional effect of paricalcitol was not accomplished by changes in blood pressure. Therefore, the most important role of active vitamin D as antiproteinuric treatment, may be in setting where up titration of RAS inhibition is hampered by too low blood pressure. Once blood pressure is normalized or low-normal and sodium intake is restricted, paricalcitol could be an option to further reduce proteinuria. As outlined, not only the effect of active vitamin D in addition to RAAS-blockade and low salt diet should be considered, but also the other CKD specific risk factors that may be influenced by vitamin D. In our clinical trial, paricalcitol treatment was accompanied by an increase in serum phosphate. Considering our concept of intertwining risk factors, we cannot rule out the possibility that this increase in phosphate levels would increase FGF23 production, besides a direct effect of active vitamin D on FGF23, ultimately nullifying the presumed beneficial effect of vitamin D when the integral risk profile is considered. Elaborating on this concept, high FGF23 levels could increase inflammatory proteins (42) which in turn increases the production of hepcidin leading to functional iron deficiency resulting in anemia. There are many studies investigating the assumed beneficial effects of vitamin D supplementation in patients with CKD but results are still contradictive indeed (43-45). However, the almost binary character of these study results makes that the effect of vitamin D on a single CKD specific risk factor is studied instead of studying the effect of vitamin D on all the CKD specific risk factors and their mutual influences on each other. Only once there is insight into the effects of vitamin D (and any other intervention) on all risk factors collectively, we will be able to identify patients that may benefit of vitamin D treatment.

Future perspectives

Despite the expanding knowledge about the interactions of CKD specific risk factors leading to novel regulating pathways, there are multiple pieces of the puzzle missing. Exploring biological effects and changes of both tissue expression and circulating levels of the whole cascade of risk factors in response to pharmacological or dietary intervention, should lead to possibilities to better modulate the risk on CVD on an individual basis for CKD patients.

In conclusion, the complex interactions between deregulated CKD specific risk factors drive cardiovascular complications and may explain why targeting the classical and CKD specific risk factors in isolation is unsatisfying in terms of prevention of complications. Current treatments, directed at CKD-specific targets, may have untoward effects on other components of the risk profile. Future studies should focus on simultaneous interventions in multiple pathways. Ultimately, accurate knowledge of these pathways will lead to patient specific risk profiles, paving the way towards personalized medicine, both at the stage of initiating treatment and its evaluation, aiming to further reduce cardiovascular complications in patients with CKD, beyond current strategies.

References

1. Hsu HJ, Wu MS. Fibroblast growth factor 23: a possible cause of left ventricular hypertrophy in hemodialysis patients. Am J Med Sci. 2009;337(2):116-22.

2. Gutierrez OM, Mannstadt M, Isakova T, Rauh-Hain JA, Tamez H, Shah A, et al. Fibroblast growth factor 23 and mortality among patients undergoing hemodialysis. N Engl J Med. 2008;359(6):584-92.

3. Tonelli M, Sacks F, Arnold M, Moye L, Davis B, Pfeffer M. Relation Between Red Blood Cell Distribution Width and Cardiovascular Event Rate in People With Coronary Disease. Circulation. 2008;117(2):163-8.

4. Felker GM, Allen LA, Pocock SJ, Shaw LK, McMurray JJ, Pfeffer MA, et al. Red cell distribution width as a novel prognostic marker in heart failure: data from the CHARM Program and the Duke Databank. J Am Coll Cardiol. 2007;50(1):40-7.

5. Forhecz Z, Gombos T, Borgulya G, Pozsonyi Z, Prohaszka Z, Janoskuti L. Red cell distribution width in heart failure: prediction of clinical events and relationship with markers of ineffective erythropoiesis, inflammation, renal function, and nutritional state. Am Heart J. 2009;158(4):659-66.

6. Pascual-Figal DA, Bonaque JC, Redondo B, Caro C, Manzano-Fernandez S, Sanchez-Mas J, et al. Red blood cell distribution width predicts long-term outcome regardless of anaemia status in acute heart failure patients. Eur J Heart Fail. 2009;11(9):840-6.

7. Emans ME, van der Putten K, van Rooijen KL, Kraaijenhagen RJ, Swinkels D, van Solinge WW, et al. Determinants of red cell distribution width (RDW) in cardiorenal patients: RDW is not related to erythropoietin resistance. J Card Fail. 2011;17(8):626-33.

8. Wolf M, White KE. Coupling fibroblast growth factor 23 production and cleavage: iron deficiency, rickets, and kidney disease. Curr Opin Nephrol Hypertens. 2014;23(4):411-9.

9. McClellan W, Aronoff SL, Bolton WK, Hood S, Lorber DL, Tang KL, et al. The prevalence of anemia in patients with chronic kidney disease. Curr Med Res Opin. 2004;20(9):1501-10.

10. McClellan WM, Flanders WD, Langston RD, Jurkovitz C, Presley R. Anemia and renal insufficiency are independent risk factors for death among patients with congestive heart failure admitted to community hospitals: a population-based study. J Am Soc Nephrol. 2002;13(7):1928-36.

11. Levin A, Thompson CR, Ethier J, Carlisle EJ, Tobe S, Mendelssohn D, et al. Left ventricular mass index increase in early renal disease: impact of decline in hemoglobin. Am J Kidney Dis. 1999;34(1):125-34.

12. Sarnak MJ, Tighiouart H, Manjunath G, Macleod B, Griffith J, Salem D, et al. Anemia as a risk factor for cardiovascular disease in The Atherosclerosis Risk in Communities (ARIC) study. J Am Coll Cardiol. 2002;40(1):27-33.

13. Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science. 2004;306(5704):2090-3.

14. Nicolas G, Chauvet C, Viatte L, Danan JL, Bigard X, Devaux I, et al. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. J Clin Invest. 2002;110(7):1037-44.

15. Merle U, Fein E, Gehrke SG, Stremmel W, Kulaksiz H. The iron regulatory peptide hepcidin is expressed in the heart and regulated by hypoxia and inflammation. Endocrinology. 2007;148(6):2663-8.

16. Isoda M, Hanawa H, Watanabe R, Yoshida T, Toba K, Yoshida K, et al. Expression of the peptide hormone hepcidin increases in cardiomyocytes under myocarditis and myocardial infarction. J Nutr Biochem. 2010;21(8):749-56.

17. Haddad S, Wang Y, Galy B, Korf-Klingebiel M, Hirsch V, Baru AM, et al. Iron-regulatory proteins secure iron availability in cardiomyocytes to prevent heart failure. European heart journal. 2017;38(5):362-72.

18. Anker SD, Comin CJ, Filippatos G, Willenheimer R, Dickstein K, Drexler H, et al. Ferric carboxymaltose in patients with heart failure and iron deficiency. N Engl J Med. 2009;361(25):2436-48.

19. Ponikowski P, van Veldhuisen DJ, Comin-Colet J, Ertl G, Komajda M, Mareev V, et al. Beneficial effects of long-term intravenous iron therapy with ferric carboxymaltose in patients with symptomatic heart failure and iron deficiencydagger. European heart journal. 2015;36(11):657-68.

20. Ge XH, Wang Q, Qian ZM, Zhu L, Du F, Yung WH, et al. The iron regulatory hormone hepcidin reduces ferroportin 1 content and iron release in H9C2 cardiomyocytes. J Nutr Biochem. 2009;20(11):860-5.

21. Kiss Z, Ambrus C, Almasi C, Berta K, Deak G, Horonyi P, et al. Serum 25(OH)-cholecalciferol concentration is associated with hemoglobin level and erythropoietin resistance in patients on maintenance hemodialysis. Nephron Clin Pract. 2011;117(4):c373-c8.

22. Patel NM, Gutierrez OM, Andress DL, Coyne DW, Levin A, Wolf M. Vitamin D deficiency and anemia in early chronic kidney disease. Kidney Int. 2010;77(8):715-20.

23. Jafar TH, Stark PC, Schmid CH, Landa M, Maschio G, Marcantoni C, et al. Proteinuria as a modifiable risk factor for the progression of non-diabetic renal disease. Kidney Int. 2001;60(3):1131-40.

24. Apperloo AJ, de Zeeuw D, de Jong PE. Short-term antiproteinuric response to antihypertensive treatment predicts long-term GFR decline in patients with non-diabetic renal disease. Kidney international Supplement. 1994;45:S174-8.

25. Eijkelkamp WB, Zhang Z, Remuzzi G, Parving HH, Cooper ME, Keane WF, et al. Albuminuria is a target for renoprotective therapy independent from blood pressure in patients with type 2 diabetic nephropathy: post hoc analysis from the Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan (RENAAL) trial. Journal of the American Society of Nephrology : JASN. 2007;18(5):1540-6.

26. Vogt L, Waanders F, Boomsma F, de Zeeuw D, Navis G. Effects of dietary sodium and hydrochlorothiazide on the antiproteinuric efficacy of losartan. Journal of the American Society of Nephrology : JASN. 2008;19(5):999-1007.

27. Mirkovic K, van den Born J, Navis G, de Borst MH. Vitamin D in chronic kidney disease: new potential for intervention. Current drug targets. 2011;12(1):42-53.

28. de Borst MH, Hajhosseiny R, Tamez H, Wenger J, Thadhani R, Goldsmith DJ. Active vitamin D treatment for reduction of residual proteinuria: a systematic review. Journal of the American Society of Nephrology : JASN. 2013;24(11):1863-71.

29. Macdougall IC, Bock AH, Carrera F, Eckardt KU, Gaillard C, Van Wyck D, et al. FIND-CKD: a randomized trial of intravenous ferric carboxymaltose versus oral iron in patients with chronic kidney disease and iron deficiency anaemia. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2014;29(11):2075-84.

30. Agarwal R, Kusek JW, Pappas MK. A randomized trial of intravenous and oral iron in chronic kidney disease. Kidney Int. 2015;88(4):905-14.

31. Bailie GR, Larkina M, Goodkin DA, Li Y, Pisoni RL, Bieber B, et al. Data from the Dialysis Outcomes and Practice Patterns Study validate an association between high intravenous iron doses and mortality. Kidney Int. 2015;87(1):162-8.

32. Miskulin DC, Tangri N, Bandeen-Roche K, Zhou J, McDermott A, Meyer KB, et al. Intravenous iron exposure and mortality in patients on hemodialysis. Clinical journal of the American Society of Nephrology : CJASN. 2014;9(11):1930-9.

33. Macdougall IC, White C, Anker SD, Bhandari S, Farrington K, Kalra PA, et al. Intravenous Iron in Patients Undergoing Maintenance Hemodialysis. The New England journal of medicine. 2018.

34. Farrow EG, Yu X, Summers LJ, Davis SI, Fleet JC, Allen MR, et al. Iron deficiency drives an autosomal dominant hypophosphatemic rickets (ADHR) phenotype in fibroblast growth factor-23 (Fgf23) knock-in mice. Proc Natl Acad Sci U S A. 2011;108(46):E1146-E55.

35. Wolf M, Koch TA, Bregman DB. Effects of iron deficiency anemia and its treatment on fibroblast growth factor 23 and phosphate homeostasis in women. J Bone Miner Res. 2013.

36. Oh HJ, Park JT, Kim JK, Yoo DE, Kim SJ, Han SH, et al. Red blood cell distribution width is an independent predictor of mortality in acute kidney injury patients treated with continuous renal replacement therapy. Nephrol Dial Transplant. 2012;27(2):589-94.

37. Eisenga MF, van LM, Leaf DE, Nolte IM, Navis G, Bakker SJL, et al. C-Terminal Fibroblast Growth Factor 23, Iron Deficiency, and Mortality in Renal Transplant Recipients. J Am Soc Nephrol. 2017;28(12):3639-46.

38. Vashistha T, Streja E, Molnar MZ, Rhee CM, Moradi H, Soohoo M, et al. Red Cell Distribution Width and Mortality in Hemodialysis Patients. Am J Kidney Dis. 2016;68(1):110-21.

39. Rygasiewicz K, Hryszko T, Siemiatkowski A, Brzosko S, Rydzewska-Rosolowska A, Naumnik B. C-terminal and intact FGF23 in critical illness and their associations with acute kidney injury and in-hospital mortality. Cytokine. 2018;103:15-9.

40. Rineau E, Gaillard T, Gueguen N, Procaccio V, Henrion D, Prunier F, et al. Iron deficiency without anemia is responsible for decreased left ventricular function and reduced mitochondrial complex I activity in a mouse model. International journal of cardiology. 2018;266:206-12.

41. Hoes MF, Grote Beverborg N, Kijlstra JD, Kuipers J, Swinkels DW, Giepmans BNG, et al. Iron deficiency impairs contractility of human cardiomyocytes through decreased mitochondrial function. European journal of heart failure. 2018;20(5):910-9.

42. Hanudel MR, Chua K, Rappaport M, Gabayan V, Valore E, Goltzman D, et al. Effects of dietary iron intake and chronic kidney disease on fibroblast growth factor 23 metabolism in wild-type and hepcidin knockout mice. American journal of physiology Renal physiology. 2016;311(6):F1369-f77.

43. Thadhani R, Appelbaum E, Pritchett Y, Chang Y, Wenger J, Tamez H, et al. Vitamin D therapy and cardiac structure and function in patients with chronic kidney disease: the PRIMO randomized controlled trial. Jama. 2012;307(7):674-84.

44. Wang AY, Fang F, Chan J, Wen YY, Qing S, Chan IH, et al. Effect of paricalcitol on left ventricular mass and function in CKD--the OPERA trial. Journal of the American Society of Nephrology : JASN. 2014;25(1):175-86.

45. Lu RJ, Zhu SM, Tang FL, Zhu XS, Fan ZD, Wang GL, et al. Effects of vitamin D or its analogues on the mortality of patients with chronic kidney disease: an updated systematic review and meta-analysis. Eur J Clin Nutr. 2017;71(6):683-93.

8

Chapter 9

NEDERLANDSE SAMENVATTING VOOR MENSEN DIE MINDER BEKEND ZIJN MET HET ONDERWERP

Nederlandse Samenvatting

Achtergrond

Chronische nierschade (CNS) komt bij 8-16% van de wereldbevolking voor en is dus een veel voorkomend gezondheidsprobleem. De meeste mensen hebben twee nieren en die hebben verschillende belangrijke functies in het lichaam. Zo zorgen de nieren ervoor dat afvalstoffen worden uitgescheiden via de urine, dat de bloeddruk netjes op peil blijft door het regelen van de vocht- en de zoutbalans en worden er meerdere hormonen geproduceerd door de nieren, zoals renine, erytropoëtine en vitamine D. Wanneer de nierfunctie meer dan drie maanden niet goed is, spreekt men van CNS. De mate van nierfalen wordt ingedeeld in 5 stadia van ernst en er zijn verschillende ziekten die nierfalen kunnen veroorzaken. Mensen die een vorm van CNS hebben, ondervinden daar doorgaans geen klachten van. Helaas is het wel zo dat het hart en de bloedvaten behoorlijke, ongunstige veranderingen doormaken gedurende het nierfalen waardoor de meeste sterfgevallen onder mensen met CNS niet worden veroorzaakt door de nierziekte zelf, maar door de gevolgen daarvan op hart- en bloedvaten. Er zijn een aantal risicofactoren geïdentificeerd die de kans op hart- en vaatziekten (HVZ) doen verhogen zoals roken, overgewicht, hoge bloeddruk of suikerziekte. Echter, deze risicofactoren blijken het verhoogde risico op HVZ bij mensen met CNS niet geheel te verklaren. En zijn dus andere, CNS-specifieke factoren zoals lekkage van eiwit in de urine (proteinurie), het optreden van ijzergebrek en bloedarmoede, stoornissen in de calcium-fosfaat huishouding/fibroblast growth factor 23 (FGF23) levels en een tekort aan vitamine D die ook een belangrijke rol lijken te spelen. Om de klinische uitkomsten van mensen met CNS te verbeteren, is het niet alleen belangrijk om de nierfunctie te beschermen, maar hen juist ook te beschermen tegen HVZ.

De huidige strategie voor het voorkomen van HVZ bij CNS patiënten is erop gericht om al deze risicofactoren apart te verbeteren middels leefstijladviezen danwel medicatie. Echter, ondanks deze aanpak blijft deze patiëntengroep een flink verhoogd risico houden op HVZ. Er zijn verschillende verklaringen voor deze onbevredigende conclusie: zo zouden de risicofactoren onderling effect op elkaar uit kunnen oefenen, zijn er hiaten in onze kennis over de pathofysiologie van HVZ bij CNS en is het effect van de medicatie die voorgeschreven wordt voor een bepaalde risicofactor mogelijk ongunstig voor een andere. Een voorbeeld is dat behandeling met actief vitamine D mogelijk een gunstig effect heeft op eiwitverlies in de urine, maar tegelijkertijd een verhoging van FGF23 kan veroorzaken, hetgeen juist weer geassocieerd is met HVZ bij CNS.

Resultaten

In dit proefschrift is gekeken naar de onderlinge effecten van de verschillende CNS specifieke risicofactoren op elkaar. Het concept hiervan wordt geïllustreerd in figuur 1 in **hoofdstuk 1**. Hierin wordt beschreven dat FGF23, ijzer en vitamine D tekort en proteïnurie belangrijke risicofactoren zijn voor het krijgen van HVZ bij patiënten met CNS. Ook leggen we uit hoe deze factoren direct en indirect zorgen voor die schadelijke effecten op het hart- en vaatstelsel. Er wordt een overzicht gegeven van wat er momenteel bekend is over de onderlinge relaties tussen deze risicofactoren en waar de hiaten in de kennis liggen. Om deze hiaten (deels) op te vullen zijn de studies uitgevoerd zoals beschreven in dit proefschrift.

In **hoofdstuk 2** hebben we gekeken naar de relatie tussen twee belangrijke afwijkingen in het bloed die kunnen zorgen voor complicaties bij CNS, nl FGF23 en red cell distribution width (RDW). RDW is een maat voor de variatie in volume van rode bloedcellen en het is een risicofactor voor het krijgen van HVZ. Als er te weinig ijzer in het lichaam aanwezig is, zal de RDW gaan stijgen. Ook heeft ijzer effect op FGF23, een hormoon dat wordt uitgescheiden door de botten en ervoor zorgt dat het fosfaatgehalte in het bloed binnen bepaalde grenzen blijft. Bij mensen met CNS wordt er veel meer FGF23 uitgescheiden dan bij mensen die een normale nierfunctie hebben. Het FGF23 dat zorgt voor het in stand houden van die fosfaatbalans noemen we de intacte vorm van FGF23 (iFGF23). Dit iFGF23 kan echter ook gekliefd worden in inactieve FGF23 fragmenten, waaronder C-terminaal FGF23 (cFGF23). FGF23 is ook een risicofactor voor HVZ, maar het is niet bekend of de verschuiving tussen de hoeveelheid iFGF23 en cFGF23 hier een rol in speelt. In dit onderzoek hebben wij gekeken bij 52 mensen met CNS en hartfalen hoe de relatie is tussen cFGF23, iFGF23 en RDW. Onze analyse toonde aan dat er een duidelijke relatie is tussen cFGF23 en RDW, maar niet tussen iFGF23 en RDW. Na correctie voor nierfunctie, fosfaat metabolisme en inflammatie parameters bleef deze relatie bestaan. Opmerkelijk hierbij is dat correctie voor ijzerstatus deze relatie niet beïnvloedt, wat suggereert dat er nog een ander mechanisme dan ijzer moet bestaan om de link tussen deze risicofactoren te verklaren.

Er zijn in het recente verleden onderzoeken gedaan waaruit blijkt dat ijzergebrek invloed heeft op de productie en klieving van FGF23. Om een beter inzicht te krijgen in het effect van ijzer op FGF23 uitscheiding en klieving, hebben wij in **hoofdstuk 3** gekeken naar het effect van verschillende ijzercondities op iFGF23 en cFGF23. Hiervoor hebben wij verschillende muizengroepen gecreëerd door bij een deel van de muizen een ijzer gebrek te bewerkstelligen middels dieet en een deel van de muizen nierinsufficiënt te maken middels het operatief wegnemen van 5/6 deel van de nieren. Ondanks een duidelijk verlaagd ijzergehalte in het bloed van de muizen met het ijzerarme dieet ten opzichte van bloed van muizen met normaal dieet, zagen wij geen verschillen

in iFGF23 en cFGF23 in gezonde muizen en bij muizen met CNS. Daarnaast is het bekend dat verschillende ijzerpreparaten zorgen voor een verschuiving in de FGF23 ratio, echter na toediening van ijzercarboxymaltose of ijzerdextraan zagen wij geen verschuiving in iFGF23 en cFGF23 levels. Om te kijken of ijzer effect heeft op de gevoeligheid voor FGF23, hebben wij recombinant FGF23 (rFGF23) toegediend. Er is gekeken of er meer fosfaatuitscheiding in de urine zou zijn door deze rFGF23 toevoeging in de verschillende muizengroepen. Echter, 24 uurs urine sparingen bij de muizen toonden geen verschil tussen groepen met een verschillende ijzerstatus. Wij hebben met deze studie geen relatie aan kunnen tonen tussen FGF23 en ijzer en zullen in de toekomst deze experimenten gaan herhalen in een muizenmodel met meer uitgesproken ijzertekort.

Renale anemie wordt voornamelijk veroorzaakt doordat de nieren minder erythropoetine uitscheiden en er minder ijzer opgenomen wordt vanuit de darm. Deze vorm van bloedarmoede komt vaker voor naarmate de nierfunctie verslechtert en is geassocieerd met het krijgen van HVZ. De reden voor verminderde ijzeropname is de verhoogde aanmaak van het stofje hepcidine door de nierziekte, waardoor de ijzerpoorten in de darm afgesloten worden. In de darm zitten namelijk kleine kanaaltjes, ferroportine, waar het ijzer door naar binnen moet, die door hepcidin gesloten worden. Wanneer het lichaam zich moet beschermen tegen teveel ijzer is dit een goed verdedigingsmechanisme, maar bij CNS zorgt het voor ijzertekort. Er komt steeds meer bewijs voor de aanname dat hepcidine ook in andere weefsels aangemaakt wordt, bijvoorbeeld in het hartweefsel. Het is nog niet opgehelderd of de productie van hepcidine op dezelfde manier gestimuleerd wordt als in de lever, de gebruikelijke productieplaats, of dat er andere factoren zijn die hepcidine productie in het hart aanzwengelen. In **hoofdstuk 4** onderzochten wij de hypothese dat cardiale hepcidine expressie wordt gestimuleerd door schade aan het hart en door de aanwezigheid van ijzer. We hebben hiervoor gekeken naar de mate van hepcidine expressie in lever en hartweefsel van ratten die een myocard infarct kregen al dan niet bij het bestaan van CNS. We zagen dat bij ratten met hartschade de hepcidine expressie hoger was dan bij gezonde ratten, maar dat er bij ratten met CNS nog meer hepcidine expressie was. Ratten die beide aandoeningen hadden, toonden de hoogste expressie. In de lever zagen we een ander expressiepatroon van hepcidine. Er bleek geen relatie tussen hepcidine en ijzer in het hart te zijn, maar wel zagen we een duidelijke relatie van cardiale hepcidine expressie en schademarkers van het hart. Wij concluderen dus dat de hepcidine expressie in het hart op een andere manier gereguleerd wordt dan in de lever en dat het onder invloed staat van schade ipv ijzer.

Naast ijzer, erythropoetine en hepcidine, speelt ook vitamine D een rol in renale anemie. In **hoofdstuk 5** wordt een overzicht gegeven van de bestaande hypothesen over de link tussen vitamine D en anemie. Uit epidemiologisch onderzoek blijkt er een duidelijke associatie te bestaan,

maar het pathofysiologische mechanisme is niet opgehelderd. De beschikbaarheid van ijzer voor bloedaanmaak zou verhoogd worden door vitamine D omdat het zowel hepcidine productie als de productie van inflammatoire cytokines onderdrukt. Daarnaast is er ook bewijs dat er directe positieve effecten zijn van vitamine D op de voorlopercellen van rode bloedcellen. We beschrijven het effect van vitamine D op HIF-1α (een regulator van EPO productie) en de relatie tussen de vitamine D receptor en de EPO receptor. Als laatste wordt de rol van een disfunctionerende bijschildklier, als gevolg van een vitamine D tekort, op het ontstaan van anemie beschreven.

Zoals eerder genoemd is eiwitverlies vanuit de nieren in de urine (proteïnurie) een belangrijke risicofactor voor het krijgen van HVZ. Bovendien kan het ook zorgen voor versnelde verslechtering van de nierfunctie. Met de huidige medicamenteuze behandeling middels zogenoemde angiotensin converting enzym blokkers (ACE inhibitors) is de proteïnurie vaak weliswaar te verbeteren, maar niet volledig te normaliseren. Van de combinatie vitamine D met lage zoutinname is bekend dat dit bij kan dragen aan vermindering van de restproteinurie. Het is echter niet bekend of het effect van vitamine D ook aanwezig is als er geen lage zoutinname is. In **hoofdstuk 6** beschrijven wij een studieontwerp waarin gekeken wordt of het effect van vitamine D op restproteinurie onafhankelijk is van zoutinname bij mensen met CNS die al de behandeling met de ACEi kregen. In **hoofdstuk 7** beschrijven wij de resultaten van deze studie waaruit blijkt dat de combinatie van verlaagde zoutinname en vitamine D suppletie het grootste effect had op het verlagen van de hoeveelheid eiwit in het bloed. Bij nadere analyse blijkt dat het eiwit verlagend effect echter vooral bewerkstelligd werd door verminderen van zoutinname.

Conclusie en implicaties

Al jarenlang proberen artsen hun patiënten met CNS te beschermen tegen HVZ door de bekende risicofactoren aan te pakken middels leefstijladviezen en/of medicijnen. Ook de zogenoemde CNS specifieke risicofactoren, zoals bloedarmoede, ijzer tekort, vitamine D tekort en restproteïnurie, worden elke apart geoptimaliseerd. Ondanks deze pogingen is de kans op overlijden aan cardiovasculaire complicaties bij deze groep patiënten erg hoog. Het besef dat deze risicofactoren elkaar ook onderling beïnvloeden zorgt ervoor dat er mogelijk nieuwe strategieën ontwikkeld kunnen worden om het risicomanagement te optimaliseren.

In dit proefschrift hebben we gekeken naar de onderlinge relaties tussen CNS specifieke risicofactoren. Als dit concept klopt, dan zou het beïnvloeden van de ene risicofactor wel eens juist een nadelig effect op een andere risicofactor kunnen hebben. Om dit concept verder te ontrafelen, zou er meer wetenschappelijk onderzoek verricht moeten worden waarbij het geïntegreerde risicoprofiel bekeken wordt. Als voorbeeld noemen wij de nog altijd voortdurende discussie over

ijzerinfusie bij CNS patiënten. Sommige studies laten zien dat ijzer gunstig is bij CNS patiënten om HVZ te voorkomen, andere studies spreken dit weer tegen. Ons voorstel zou zijn om in dergelijke studies ook de iFGF23 en cFGF23 levels te meten, naast andere componenten van een mogelijk veranderend risicoprofiel zodat er gekeken kan worden of, hoe en bij wie ijzer deze concentraties beïnvloedt. Een onvoordelige shift in iFGF23/cFGF23 ratio bijvoorbeeld, zou misschien wel het variabele effect van ijzerinfusie op het cardiovasculaire risico kunnen verklaren. Een ander voorbeeld is het voorschrijven van vitamine D aan CNS patiënten. Zoals wij beschreven hebben zou vitamine D gunstige effecten hebben op vermindering van restproteïnurie, echter vitamine D zorgt ook voor een verhoging van FGF23 waardoor het risico op HVZ juist weer toeneemt.

Concluderend weten we tegenwoordig steeds meer over de wisselwerking van de CNS specifieke risicofactoren op elkaar, maar zijn de complexe interacties nog niet volledig opgehelderd. Toekomstige studies zouden zich moeten richten op een geïntegreerde interventie in meerdere routes met behulp van leefstijlinterventies en/of medicijnen. Als deze complexe pathways volledig opgehelderd zijn dan kunnen we in de toekomst patiënt specifieke risicoprofielen opstellen waardoor we mensen met CNS zo optimaal mogelijk kunnen beschermen tegen complicaties in hart en bloedvaten.

9

Appendix

DANKWOORD

Dankwoord

Toen ik begon met promoveren werd ik door verschillende mensen ‘gewaarschuwd’ voor het doen van onderzoek. Ik hoorde ‘je bent daar niet het type voor’, ‘ga je dat echt doen tijdens je opleiding tot internist?’ en ‘het is echt een uitputtingsslag die artikelen schrijven’. Maar ik besloot het toch te doen. Om aan mezelf te bewijzen dat ik het kon. Om te ervaren of ik het leuk zou vinden....

Wat ben ik blij dat ik mijn hart gevolgd heb! Want ondanks dat het inderdaad een uitdagende en stressvolle tijd was door de verschillende ballen die ik zowel zakelijk als privé in de lucht moest houden, heeft het mij persoonlijk erg veel gebracht. Echter, het zou allemaal niet gelukt zijn zonder de mensen om mij heen. Wat heb ik een steun ervaren, alleen dat al was het promoveren meer dan waard!

Allereerst wil ik mijn promotor Prof.dr. Vervloet bedanken. Beste Marc, wat ben je toch goed in wat je doet! Als ik even vastzat kon jij mijn gedachten weer ordenen door een paar heldere overzichtsplaatjes te schetsen. Echt, zonder jou was dit allemaal niets geworden. Ik bewonder je enorm en ben blij dat we collega’s blijven. Zo hoop ik stiekem nog heel veel van je te leren en de kunst nog meer van je af te mogen kijken. Dank, voor alles.

Professor C.A.J.M. Gaillard, beste Carlo: dank je wel voor het vertrouwen dat je in mij had om te starten met een project over ijzer en FGF23. Ik vergeet nooit dat je naar mij toe kwam om te vertellen dat je naar Groningen ging. Stress alom! Gelukkig hebben we ondanks de afstand altijd goed kunnen samenwerken en heeft dit toch binnen redelijke tijd geleid tot dit boekje. Inmiddels werk je in Utrecht: iets dichterbij en hopelijk blijven we elkaar zo nu en dan spreken.

Dr. M.H. de Borst, beste Martin: jij bent in mijn promotieteam gekomen toen Carlo naar Groningen ging. We hebben elkaar niet heel veel gezien, maar jouw prikkelende opmerkingen bij mijn stukken hebben mij een stuk wijzer gemaakt. Dank je wel hiervoor. Succes met alle mooie projecten die je hebt lopen in Groningen en veel geluk met je gezin.

De leden van mijn promotiecommissie: Prof.dr. M. Kramer, Prof.dr. P. Evenepoel, Prof.dr. J. van der Velden, dr. B. Braam, dr. L. Vogt en dr. J.H. van der Heijden, hartelijk dank voor uw beoordeling van dit proefschrift. Dr. B. Braam, beste Branko, jou wil ik in het bijzonder bedanken voor de bijdrage aan mijn hepcidine artikel en voor het feit dat je vanuit Canada overgekomen bent om aanwezig te zijn bij de verdediging van mijn proefschrift.

Hoewel niet in mijn officiële promotieteam maar o zo waardevol geweest in deze jaren als afdelingshoofd: Prof.dr. F.J. van Ittersum. Beste Frans, wat geef jij mij een gevoel van vertrouwen. Altijd kan ik je kamer binnenlopen voor een heerlijk nuchter maar zeer genuanceerd en doordacht advies. Op dagen dat ik van jou thuis mocht schrijven heb ik flinke meters kunnen maken.

Alle collega's van de afdeling nefrologie: Muriel Grooteman, Joost van der Heijden (wat leuk dat je mijn opponent bent!), Azam Nurmohamed, Brigit van Jaarsveld, Wim Ruger, Piet ter Wee, Annet Bouma, Janneke Rood, Catherine Jurgens, Yu-Sok Kim, Frederiek Heenan en de aanstormende bijna klare nefrologen: het voelt als een voorrecht om te mogen werken in zo'n bevlogen team waar ik me mag ontwikkelen op gebied van klinisch werk, onderzoek en management. Dank jullie wel voor de gezelligheid en collegialiteit. Muriel, als kamermaatje had ik het niet beter kunnen treffen: op onze 2 m2 wordt het harde werken regelmatig afgewisseld met het delen van ons wel en wee. Dank voor je oprechte interesse. Ook alle verpleegkundigen van de afdeling nefrologie VUmc, Niercentrum aan de Amstel en Diapriva wil ik bedanken voor de gezelligheid en de fijne samenwerking. Hopelijk blijft ons team net zo hecht als nu na de fusie met het AMC.

Bij aanvang van dit promotieonderzoek hebben Carlo en Marc mij wel een beetje in het zadel gehesen door mij in contact te brengen met Mireille Emans, Lennart Bongartz, Charlotte te Velde-Keyzer, Melissa Verkaik en Jaap Joles. Dank jullie wel voor jullie kennis en kunde, ik heb veel van jullie mogen leren.

Eelco Keuning: mijn muizenheld! Jij weet echt alles over de DEC, ongerief, de 3 V's, verdunningen etc. Met heel veel geduld heb jij mij geholpen met het opzetten en uitvoeren van mijn muizenstudie. Uren hebben we samen zitten sparren, etiketjes plakken en boven muizenstaartjes gehangen om die druppels bloed te oogsten. Samen met Rika en de andere medewerkers van het Universitair Proefdier Centrum hebben jullie mij door de meest angstige momenten heen geloodst. Want helaas, de angst voor muizen heb ik nog steeds..... Dank je wel voor alles.

Mijn vroegere kamergenoten in 'het kippenhok'. Camiel en Aaltje; dank jullie wel voor de gezellige tijd en het luisteren naar mijn 'blonde vragen en opmerkingen'. Tiny, met veel geduld maakte jij mij wegwijs in SPSS al raakten we regelmatig in de war van al die groepen muizen. Dank je wel voor al je geduld.

Als je hard werkt, heb je ook genoeg ontspanning nodig. Mijn vriendinnen zorgden daar wel voor. Lieve Jamila, Nicole, Marloes, Sanne, Avital, Agnes, Anouk, Ingeborg en Suzanne: dank jullie wel voor alle gezellige wandelingen, lunches, weekendjes weg, maandborrels en serieuze gesprekken.

Door jullie word ik eraan herinnerd dat het leven niet alleen maar uit werken bestaat. Op naar heel veel leuke uitjes met elkaar!

En dan onze lieve oppassen. Maartje, jij bent vanaf het begin betrokken bij ons gezin. Ik kan je niet vaak genoeg zeggen hoe dankbaar wij zijn voor alle hulp en liefde die jij in de beginjaren gegeven hebt. Rosanne en Lisette, door jullie ga ik elke dag zonder zorgen naar mijn werk. Duizendmaal dank voor alles wat jullie doen om ons gezin draaiende te houden. Jullie zijn mijn steun en toeverlaat.

Ook een woord van dank aan mijn schoonouders Ria en Leo. Onvermoeibaar kwamen jullie jarenlang elke vrijdag naar Lisse om op onze meiden te passen. Door jullie goede zorgen kon ik vrijdagmiddag vaak nog even doortrekken en net dat stukje afmaken waar ik mee bezig was. Om dan in een heerlijk opgeruimd huis thuis te komen met gewassen meisjes en een lekkere maaltijd. Jullie hulp tijdens de verhuizing was ongelofelijk. Dank jullie wel!

Pap en mam: jullie hebben mij gemaakt tot wie ik ben. Wat hebben jullie ons een heerlijke jeugd gegeven en een stevige basis. Vanuit die basis is het fijn uitvliegen! Om regelmatig weer even op het nest terug te komen voor een dagje schrijven, lunchen met zijn allen, een kopje thee of gewoon even kletsen. Ik gun jullie de komende jaren veel mooie reizen met jullie nieuwe camper, geniet ervan!

En dan mijn zusjes en paranymfen. Rieke, ik leer van jou hoe je zelf de slingers op kunt hangen in het leven. Je geeft mij vaak net even het laatste zetje om iets voor mezelf te gaan doen in plaats van altijd maar doorwerken. Dank je wel daarvoor. Arda, heerlijk om soms samen te sparren over patiënten en het moederschap. Hopelijk blijft jullie nieuwe aanwinst tot op de uitgerekende datum zitten want ik zou het fantastisch vinden om jou naast mij te hebben op deze voor mij zo belangrijke dag. Ik zeg het niet vaak, maar ik ben ongelofelijk trots dat jullie mijn zusjes zijn.

Teske, Bente, Mille. Jullie zijn mijn alles, dat weten jullie. Wat een vreugde, geluk, zingeving (maar ook drukte) brengen jullie in ons leven! Ik ben super trots op mijn nieuwsgierige, slimme, sociale en liefste kinderen van de wereld. Ik hoop dat jullie mij vergeven dat ik soms tijd aan mijn onderzoek moest besteden in plaats van aan jullie. Ik hoop dat jullie mijn promotie, zeker later, zullen zien als motivatie om je ambities waar te maken. Ook al denken anderen, en jezelf wellicht ook, dat je het niet kunt. En dat het combineren van een carrière en het hebben van een prachtig gezin gewoon mogelijk is met een gezonde dosis doorzettingsvermogen. Meiden, we gaan er mooie jaren van maken samen!

Lieve Rick, de allerlaatste woorden van dit proefschrift zijn voor jou. Dank je wel dat je mij zo gelukkig maakt. De enige man in ons gezin, zo nuchter als maar kan en niet van zijn stuk te brengen. Wat heb ik een ongelofelijk respect voor hoe jij in het leven staat en wat vullen we elkaar goed aan. Zonder jou zou ik de helft nog niet zijn van wat ik nu ben. Met heel mijn hart hoop ik op een voortzetting van onze mooie jaren samen. Voor altijd.....